이학박사학위논문

비만에서 지방조직의 염증반응 조절에 관한 Hypoxia-Inducible Factor 2α의 기능연구

Role of Hypoxia-Inducible Factor 2α in the Regulation of Adipose Tissue Inflammation in Obesity

2014년 2월

서울대학교 대학원

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최성식

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이 논문을 이학박사 학위논문으로 제출함

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2014 년 2 월

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ABSTRACT

Role of Hypoxia-Inducible Factor 2α in the Regulation of Adipose Tissue Inflammation in Obesity

Sung Sik Choe

Excess caloric intake leads to expansion of adipose tissue, which induces adipose tissue hypoxia because of the relative insufficiency of the vascular network in charge of oxygen supply. In obesity, adipose tissue hypoxia has been implicated be one of important mediator of pro-inflammatory responses, which is associated with obesity-related metabolic complications. Recently, hypoxia-inducible factor (HIF) family, which is a major transcription factor induced by hypoxia, is suggested to play essential roles to mediate hypoxic responses in adipose tissue. For instance, activation of HIF-1α has been implicated in chronic inflammation, fibrosis, and insulin resistance as well as hypoxia-mediated angiogenesis in obese adipose tissue. However, the pathophysiological role of HIF-2α in adipose tissue has not been thoroughly elucidated in obesity. Evidence has suggested that HIF-2α would have its own physiological roles or opposing roles compared to HIF-1α despite of their extensive homology. Therefore, HIF-2α is also proposed to have distinctive roles in the regulation of adipose tissue angiogenesis and inflammation in obesity.

In this study, I demonstrate that activation of HIF-2α in adipocytes could increase the expression level of pro-angiogenic factors as well as recruit

macrophage. In the adipose tissue of short-term high-fat diet (HFD)-fed mice, adipocyte HIF-2α mRNA and protein were rapidly induced. In addition, the overexpression of HIF-2α in adipocytes significantly increased the expression level of pro-angiogenic factors such as VEGFα, and ANGPTL4 regardless of hypoxic condition. On the other hand, HIF-2α-overexpressing adipocytes promoted the recruitment and migration of macrophages, which were directly and indirectly mediated by nitric oxide signaling. Interestingly, macrophages co-cultured with adipocytes exhibited increased expression level of pro-angiogenic factors such as IGF and PDGFβ. Furthermore, macrophages sensitively and drastically stimulated the expression of VEGFα and AMD mRNA more than adipocytes in chronic hypoxic condition. These results suggest that macrophage recruited by adipocyte HIF-2α might participate in adipose tissue angiogenesis.

In addition, I demonstrate that elevation of macrophage HIF-2α would attenuate adipose tissue inflammation and improve insulin resistance in obesity. In macrophages, overexpression of HIF-2α decreased nitric oxide production and suppressed expression of pro-inflammatory cytokines through induction of arginase 1 (ARG1). HIF-2α-overexpressing macrophages alleviated pro-inflammatory responses and improved insulin resistance in adipocytes. In contrast, knock-down of macrophage HIF-2α promoted palmitate-induced pro-inflammation responses in adipocytes. Furthermore, compared to wild-type mice, HIF-2α heterozygous-null mice aggravated insulin resistance and adipose tissue inflammation with more M1-like ATMs upon HFD. Moreover, glucose intolerance in HFD-fed HIF-2α

heterozygote mice was relieved by macrophage deletion with clodronate treatment. Thus, unlike HIF-1α, HIF-2α in ATMs is newly suggested to contribute to the resolution of adipose tissue inflammation and insulin resistance in obesity.

Taken together, these data suggest that in early stage of obesity having minimal inflammation, HIF-2α might regulate adipose tissue remodeling to adapt to the metabolic environment changes through regulation of angiogenic genes and suppression of excessive pro-inflammatory responses. Therefore, appropriate activation of HIF-2α would be crucial to maintain homeostasis of adipose tissue function and vasculature, leading to metabolically healthy adipose tissue expansion.

Key words: Adipose tissue hypoxia, Adipose tissue inflammation, Adipose tissue macrophage, Adipose tissue remodeling, Hypoxia-Inducible Factor 2α, Insulin resistance, Obesity

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BACKGROUND

1. Obesity, diabetes, and inflammation

1) Obesity and adipose tissue inflammation

Accumulating evidences have suggested that obesity is characterized by chronic and low-grade inflammatory state, which is closely associated with metabolic complications such as insulin resistance, type 2 diabetes, cardiovascular disease, musculoskeletal disorders, and certain cancers (Donath and Shoelson, 2011; Olefsky and Glass, 2010; Wellen and Hotamisligil, 2003). In obesity, adipose tissue appears to be the primary tissue to initiate inflammatory responses upon excess nutrients and sensitively to respond to various inflammation inducing factors (Donath and Shoelson, 2011; Olefsky and Glass, 2010; Wellen and Hotamisligil, 2003). For instance, saturated free fatty acids (FFAs), known as a dietary risk factor, work as potent toll-like receptor 4 (TLR4) activators in adipocytes because of their structural similarities to bacteria-derived lipids such as lipopolysaccharides (LPS) (Saberi et al., 2009; Shi et al., 2006; Suganami et al., 2007). As the TLR4 signaling is one of well-known innate immune pathways, activation of TLR4 by saturated FFAs directly stimulates JUN N-terminal kinase (JNK) and nuclear factor-κB (NFκB) signaling pathways. This subsequent activation process stimulates gene expression of pro-inflammatory cytokines including tumor necrosis factor α (TNFα), interleukin-1beta (IL-1β), IL-6, and IL-12 in adipose tissue of obesity (Saberi et al.,

2009; Shi et al., 2006; Suganami et al., 2007). In addition, saturated FFAs induce ER stress, which relates to activate several downstream signaling pathways including JNK1 (Arkan et al., 2005; Hirosumi et al., 2002; Shoelson et al., 2006; Solinas et al., 2007). Moreover, expansion of adipose tissue in obesity leads to relative deficiency of vascular supply, resulting in adipose tissue hypoxia, which also triggers inflammatory activation (Goossens and Blaak, 2012; Hosogai et al., 2007; Rausch et al., 2008; Wood et al., 2009; Ye et al., 2007). Hypoxia-mediated pro-inflammatory responses will be discussed later.

2) Inflammation and insulin resistance

Many evidences have shown that pro-inflammatory cytokines, such as TNFα, L1-β, IL-6, and IL-12 released by adipocytes, are linked to insulin resistance in liver, muscle, heart, as well as adipose tissue (Donath and Shoelson, 2011; Olefsky and Glass, 2010; Wellen and Hotamisligil, 2003). Recently, it has been revealed that activation of IκB kinase-β (IKKβ) and JNK is one of the mechanisms by which pro-inflammatory cytokines repress insulin action in metabolic organs. Activation of these stress-activated kinases induces serine phosphorylation of insulin receptor substate 1 (IRS1) and IRS2, the key downstream mediators of insulin receptor, and thus interferes with normal insulin action, subsequently leading to insulin resistance (Chawla et al., 2011; Ferrante, 2007; Olefsky and Glass, 2010; Tilg and Moschen, 2008). In addition, IKKβ and JNK augment transcriptional activity of pro-inflammatory transcription factors such as activator protein 1 (AP1)

and NFκB to further stimulate expression of pro-inflammatory genes, which aggravate insulin resistance (Odegaard and Chawla, 2011; Wellen and Hotamisligil, 2005). In accordance with these, ablation or inhibition of IKKβ or JNK improves insulin resistance and pro-inflammatory responses in *in vivo* and *in vitro* model systems (Austin et al., 2008; Hirosumi et al., 2002; Nguyen et al., 2005; Wellen and Hotamisligil, 2005; Yuan et al., 2001; Zhang et al., 2011)

3) Adipose tissue macrophages (ATMs)

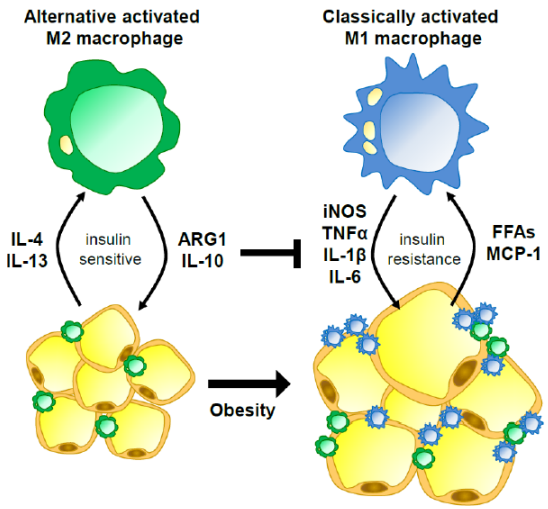
Adipose tissue is not only energy storage organ to stock up on surplus nutrients but also endocrine organ to secrete adipokines such as leptin, adiponectin, and inflammatory cytokines for active control of metabolic homeostasis. Among various cell types in adipose tissue, adipocytes play a primary role for metabolic regulation and other cells including macrophages are also involved in transporting nutrients and secreting adipokines required for whole body energy homeostasis (Lumeng et al., 2007b; Weisberg et al., 2003; Xu et al., 2003). Thus, the number and activation state of adipose tissue macrophages (ATMs), actively cross-talk with adipocytes in adipose tissue, reflect the metabolic status. For example, in lean mice, ATMs comprise about 10% of stromal vascular cells (SVCs) and they show alternatively activated (M2) phenotype. However, in the progress of obesity, macrophage content increases up to about 50% of SVCs, and ATMs are polarized from M2 type to classically activated (M1) type (Fujisaka et al., 2009; Lumeng et al., 2007a; Sun et al., 2011). The shifting balance between M1 and M2 ATMs is

important to determine the level of inflammatory tones in adipose tissue of obesity (Fig. 1). In addition to the contribution of accumulated ATMs in adipose tissue to the development of insulin resistance, recent studies have suggested that these cells also participate in adipose tissue remodeling for adaptation to metabolic environments (Odegaard and Chawla, 2011; Wynn et al., 2013).

In obesity, pro-inflammatory blood monocytes are recruited and infiltrated into adipose tissues and differentiate into $CD11c^{+}$ pro-inflammatory macrophages which often form crown-like structures (CLSs) around dead adipocytes. (Lumeng et al., 2007a; Weisberg et al., 2006; Weisberg et al., 2003). Polarization of ATMs into M1 type attributes to various changes of local microenvironments such as saturated free fatty acids (FFAs), ER stress, and tissue hypoxia (Sun et al., 2011). In particular, monocytes chemoattractant protein-1 (MCP-1) secreted from obese adipose tissue is known to play crucial roles in recruitment of pro-inflammatory blood monocytes via C-C motif chemokine receptor-2 (CCR2). Accordingly, it has been reported that CCR2 deficient mice exhibit low level of pro-inflammatory ATMs, which is sufficient to protect mice against diet-induced insulin resistance (Lumeng et al., 2007a; Weisberg et al., 2006). In contrast, adipose tissue-specific MCP-1-overexpressing mice show increased adipose tissue inflammation and insulin resistance upon high-fat diet (HFD) (Kamei et al., 2006). Therefore, accumulation of pro-inflammatory M1 ATMs in adipose tissue is an crucial step for obesity-induced insulin resistance and pathogenesis of metabolic diseases.

On the other hands, M2 ATMs play a key role to repair adipose tissue

Figure 1. Cross-talk between adipocytes and macrophages plays an important role in insulin sensitivity in adipose tissue. Under lean conditions, adipocytes secrete Th2 cytokines such as IL4 and IL13 that promote alternative activation of macrophages. Alternatively activated (M2) macrophages suppress pro-inflammatory response in adipocytes through secretion of anti-inflammatory cytokines or scavenging nitric oxide via arginase 1 (ARG1) activation. Meanwhile, under obese conditions, hypertrophic adipocytes promote recruitment of classically activated (M1) macrophages via release of free fatty acids (FFAs) and secretion of monocyte chemotactic protein 1 (MCP1). Activated M1 macrophages produce large amounts of pro-inflammatory cytokines such as TNFα, IL-1β, IL6 as well as nitric oxide via iNOS activation, which act on adipocyte to induce insulin resistance. This vicious cycle further amplifies inflammation and insulin resistance.


Alternative activated
M2 macrophage
Classically activated
M1 macrophage
IL-4
IL-13
insulin
sensitive
ARG1
IL-10
iNOS
TNFα
IL-1β
IL-6
insulin
resistance
FFAs
MCP-1
Obesity

damages induced by pro-inflammatory M1 macrophages and to maintain insulin sensitivity for metabolic homeostasis (Fujisaka et al., 2009; Odegaard and Chawla, 2011; Shaul et al., 2010). Furthermore, M2 ATMs exhibit high levels of anti-inflammatory cytokines such as IL-10 and specific enzymes such as arginase 1 (ARG1), accompanied with low levels of pro-inflammatory signals (Fujisaka et al., 2009; Lumeng et al., 2007a), which contributes to repress adipose tissue inflammation (Fujisaka et al., 2009; Lumeng et al., 2007a; Sun et al., 2011). Adipose tissue secrets Th2 cytokines such as IL-4 and IL-13, which retains alternative activation of ATMs, and alternative activation of M2 ATMs by Th2 cytokines relieves metabolic dysregulation and restores insulin sensitivity in obesity (Kang et al., 2008; Odegaard et al., 2008; Ricardo-Gonzalez et al., 2010). Consistently, dysregulation of alternative M2 activation develops insulin resistance through augmentation of pro-inflammatory responses of ATMs in adipose tissue of obesity (Kang et al., 2008; Odegaard et al., 2007).

2. Adipose tissue hypoxia

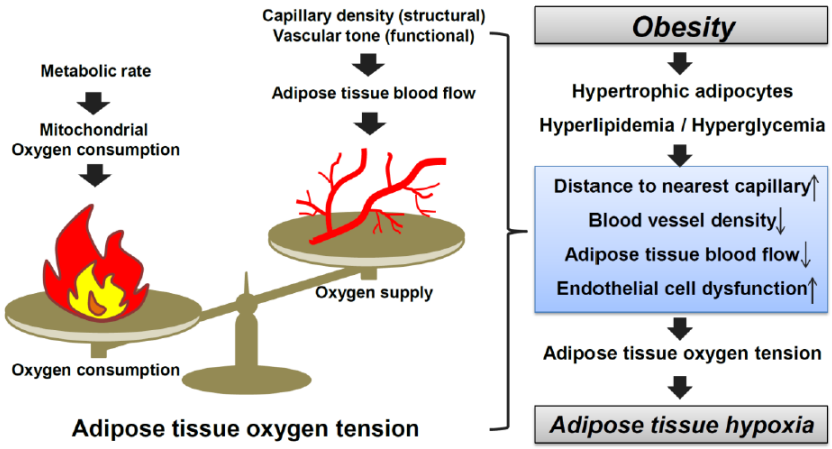
1) Adipose tissue hypoxia in obesity

Compared to other tissues and organs, adipose tissue has a unique character. The size and growth of adipose tissue are very flexible and dynamically changed in response to nutrient status (Lee et al., 2011b; Sun et al., 2011). In normal development of adipose tissue, adipose tissue expansion accompanies with

angiogenesis for maintenance of vascular homeostasis, which serves to transport nutrients and adipokines for proper functional performance of adipose tissue (Cao, 2007; Hausman and Richardson, 2004; Rutkowski et al., 2009; Yilmaz and Hotamisligil, 2013). However, in pathological expansion such as obesity, rapidly enlarged adipocytes so called hypertrophic adipocytes lead to imbalance between supply and consumption of oxygen because of the relative insufficiency of vascular networks. Thus, deficient oxygen tension in obese adipose tissue induces local hypoxic environments (Hosogai et al., 2007; Sun et al., 2011; Wood et al., 2009; Ye et al., 2007). Also, reduction of adipose tissue blood flow and/or blood perfusion in obesity further aggravates adipose tissue hypoxia (Andersson et al., 2012; Arngrim et al., 2013; Goossens et al., 2011; Goossens and Blaak, 2012) (Fig. 2).

The direct evidences for adipose tissue hypoxia in obese animal models have been recently reported (Hosogai et al., 2007; Rausch et al., 2008; Ye et al., 2007). For instance, it has been shown that partial pressure of O_2 in adipose tissue of obese mice is decreased than lean mice (Rausch et al., 2008; Ye et al., 2007). In epididymal adipose tissues (EATs) of *ob/ob* mice that is a well-characterized severe obese mouse model, O_2 pressure is about one-third of that in the control mice (15.2 mmHg in *ob/ob* vs. 47.9 mmHg in lean control), whereas in vein blood of *ob/ob* mice, O_2 pressure is not significantly different from that in the control mice (23 mmHg). Reduction of O_2 pressure to 15.2 mmHg in the adipose tissue is chronic hypoxic condition with 2% O_2 concentration. Also, it has been demonstrated that the signal of chemical hypoxic probe, pimonidazole, is elevated in adipose tissue of

Figure 2. Balance between supply and consumption of oxygen determines adipose tissue hypoxia. Oxygen pressure in adipose tissue results from the balance between oxygen supply and oxygen consumption. Impairment of this homeostatic balance in adipose tissue leads to pathological condition such as adipose tissue hypoxia. In obesity, adipose tissue hypoxia is responsible for several factors, such as decrease of blood vessel density and blood flow and endothelial cell dysfunction in adipose tissue.


Capillary density (structural)
Vascular tone (functional)
Adipose tissue blood flow
Metabolic rate
Mitochondrial
Oxygen consumption
Oxygen supply
Oxygen consumption
Adipose tissue oxygen tension
Obesity
Hypertrophic adipocytes
Hyperlipidemia / Hyperglycemia
Distance to nearest capillary
Blood vessel density
Adipose tissue blood flow
Endothelial cell dysfunction
Adipose tissue oxygen tension
Adipose tissue hypoxia

obese mice (Hosogai et al., 2007; Rausch et al., 2008; Ye et al., 2007). The hypoxia probe reacts with proteins in a low-oxygen environment under 10 mmHg. Furthermore, the expression of hypoxia response genes are increased in adipose tissue of obese mice (Hosogai et al., 2007; Rausch et al., 2008; Ye et al., 2007). In obese adipose tissue, the expression of hypoxia response genes including hypoxia-inducible factor α (HIFα), vascular endothelial growth factor α (VEGFα), glucose transporter 1 (GLUT1), Heme oxygenase 1 (HO-1), and pyruvate dehydrogenase kinase 1 (PDK1) are significantly promoted as compared to lean adipose tissue. However, the expression levels of these genes are not elevated in other tissues including muscle or liver of obese mice. In accordance with these, the level of lactate in adipose tissue of obese mice is higher than that of lean mice (Hosogai et al., 2007). Because ATP is generated by glycolytic process without O_2 consumption under hypoxia, production of lactate, final product of glycolytic reaction, is augmented. In adipose tissue of obese mice, lactate concentration is about 1.5 fold higher than in that of lean mice.

2) Adipose tissue hypoxia and angiogenesis

In rapidly expanding adipose tissue, hypoxia is a important factor for angiogenesis as the case of solid tumor propagation (Rutkowski et al., 2009). In obesity, adipose tissue hypoxia stimulates accumulation of HIF-1α protein, a master transcription factor mediating hypoxic responses in adipocytes, potentially leading to the up-regulation of angiogenic factors such as VEGFα, leptin, adrenomedullin

(ADM), and plasminogen activator inhibitor-1 (PAI-1) (Rutkowski et al., 2009; Ye, 2009; Ye et al., 2007). It has been shown that hypoxia or chemical inducers of cellular hypoxia such as $CoCl_2$ stimulates VEGFα and leptin gene expression in adipocytes. Consistently, promoter activity and induction of VEGFα and leptin genes are abolished by HIF-1α knock-down using siRNA (Ambrosini et al., 2002; Grosfeld et al., 2002; He et al., 2011; Wang et al., 2007; Zhang et al., 2010). In addition, it has been recently demonstrated that the tip region of EATs is extremely hypoxic and expresses high level of angiogenic factors including VEGFα, implying that hypoxia-mediated angiogenesis is also important in expansion of adipose tissue (Cho et al., 2007). Therefore, in adipose tissue, hypoxia appears to be one of physiological signals for angiogenesis in both physiological and pathological condition.

3) Adipose tissue hypoxia and inflammation

It has been proposed that adipose tissue hypoxia in obesity would be one of key factors to produce inflammation-related adipokines in adipocytes (Hosogai et al., 2007; Rausch et al., 2008; Wood et al., 2009; Ye et al., 2007). In physiological condition, adipocyte producing inflammatory cytokines responding to hypoxia are expected to mediate acute increase of blood flow and chronic stimulation of angiogenesis together with angiogenic signals for adipose tissue development and remodeling (Cao, 2007). However, in pathological conditions such as obesity, chronic increase of inflammatory cytokines in hypoxic adipose tissue seem to

contribute to adipose tissue inflammation linked to insulin resistance (Goossens and Blaak, 2012; Wood et al., 2009; Ye, 2009).

The effect of hypoxia on the regulation of adipocytokines has been tested with *in vitro* culture system. Many studies have demonstrated that acute exposure to severe hypoxic condition (1% O_2) induces the expression of pro-inflammatory cytokines including TNFα, IL-1β, IL-6, MCP-1, PAI-1, and macrophage migration inhibition factor (MIF), whereas it reduces the expression of adiponectin, one of anti-inflammatory cytokines, which is mediated by activation of several transcription factors such as HIF-1α and NFκB (Chen et al., 2006; Hosogai et al., 2007; Mazzatti et al., 2012; Wang et al., 2007; Wree et al., 2012; Ye et al., 2007; Yu et al., 2011). Among pro-inflammatory cytokines, MCP-1 is critical to attract macrophage to adipose tissue (Kamei et al., 2006; Kanda et al., 2006; Weisberg et al., 2003). Meanwhile, MIF induced by adipose tissue hypoxia is involved in the inhibition of macrophage departure from the hypoxic region (Turner et al., 1999), consequently accelerating macrophage accumulation in obese adipose tissue.

Adipose tissue hypoxia also affects other cells such as preadipocytes, endothelial cells, and immune cells including macrophages. Among these cells, recruited macrophages are one of major contributors in production and release of many pro-inflammatory cytokines and chemokines in adipose tissue (Fain, 2006; Fain et al., 2004). In a recent study, immunohistochemical evidence indicates that pro-inflammatory macrophages are primarily located in hypoxic area in obese adipose tissue (Rausch et al., 2008). In addition, it has been suggested that

macrophages under hypoxia cause adipose tissue dysfunction via the secretion of pro-inflammatory cytokines (Ye et al., 2007). Therefore, adipose tissue hypoxia promotes not only macrophage accumulation but also stimulates pro-inflammatory responses in recruited macrophages, leading to aggravation of chronic inflammation and insulin resistance in obesity.

4) Adipose tissue hypoxia and adipocyte dysfunction

Adipose tissue hypoxia has been implicated in chronic inflammation in obese adipose tissue. Recent studies have suggested that hypoxia is a potential risk factor to induce several other stress conditions such as lipotoxicity, ER stress, and mitochondrial dysfunction, which are involved in adipocyte dysfunction in obese adipose tissue. For example, adipose tissue hypoxia increases FFAs release in adipocytes via adipocyte necrosis, inhibition of peroxisome proliferator-activated receptor γ (PPARγ), and AMPK activation (Liu et al., 2006; Yin et al., 2009; Yun et al., 2002), which mediates lipotoxicity in metabolic organs (Yin et al., 2009). Also, it has been reported that ER stress is induced by hypoxia in adipocytes (Hosogai et al., 2007). The mRNA expressions of CHOP and GRP78 as well as phosphorylation of eIF2a, which are well-defined ER stress markers, are increased in adipocytes by hypoxia. Moreover, it has been shown that activation of HIF-1α mediates the inhibition of mitochondrial function in adipocytes (Kim et al., 2006; Zhang et al., 2007). Thus, it is very likely that adipose tissue hypoxia is tightly associated with adipocyte dysfunction and death in obesity, which eventually lead to insulin

resistance and obesity-associated diseases.

3. Hypoxia-inducible factor α

1) Hypoxia-inducible factor family

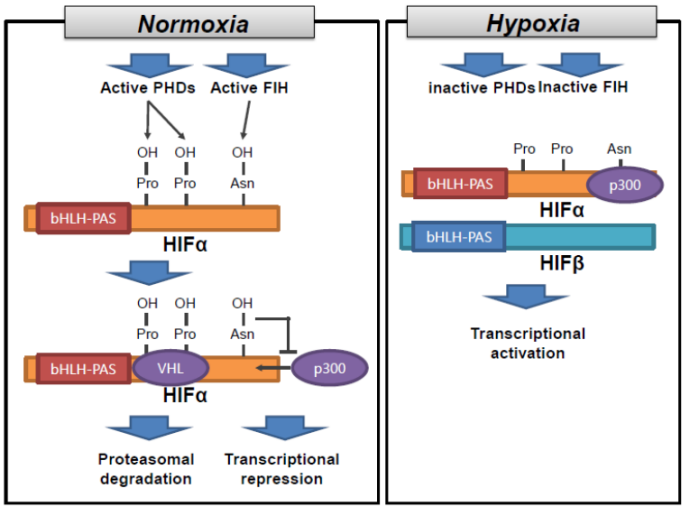
Hypoxia-inducible factor (HIF) family is the major transcription factor to mediate hypoxic response (Kaelin and Ratcliffe, 2008; Majmundar et al., 2010; Semenza, 1999). HIFs are basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) proteins that consist of three oxygen-sensitive α-subunit isoforms (HIF-1α, HIF-2α/endothelial PAS protein 1 (EPAS1), and HIF-3α), which forms protein complex with stable HIF-1β/aryl hydrocarbon receptor nuclear translocator (ARNT). HIF heterodimers bind to hypoxia-responsive elements (HREs). Under hypoxia, three HIFα isoforms are regulated by post-translational modification-mediated stabilization (Kaelin and Ratcliffe, 2008; Majmundar et al., 2010) (Fig. 3). In normoxic condition, HIFα subunits are modified by HIF-specific prolyl-hydroxylases (PDHs), followed by proteasomal degradation through interaction with the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex. Meanwhile, asparaginyl hydroxylation by factor inhibiting HIF (FIH) blocks coactivator recruitment. It has been reported that many of HIF target genes identified, which are associated with O_2 homeostasis including angiogenesis, blood vessel remodeling, erythropoiesis, and glycolysis as well as developmental processes such as stem cell identity, cell differentiation, cell cycle, and cell growth (Patel and Simon, 2008;

Ratcliffe, 2007; Semenza, 1999). In addition, functional roles of HIF family are concerned with patho-physiological condition such as various cancers, arthritis, and obesity (Keith et al., 2012; Semenza, 1999; Sun et al., 2011; Wood et al., 2009). Despite of these findings for HIF family, it is required to define the cell- or tissue-specific functional roles of HIF family in various cell or tissue types.

2) HIF-1α and HIF-2α

HIF-1α has been first identified by affinity purification using promoter of a hypoxic response gene, erythropoietin (EPO) (Wang et al., 1995). While HIF-2α and HIF-3α have been cloned by homology search with HIF-1α, HIF-1β has been discovered by screening for interaction partners. Among three HIFα isoforms, HIF-1α and HIF-2α have been extensively investigated and known to be very closely related in terms of structure, reactivity to hypoxia, HRE-dependent gene transcription, and their target genes such as VEGFα, GLUT1 and adrenomedullin (ADM) (Patel and Simon, 2008). However, despite of extensive homology between HIF-1α and HIF-2α, they have distinct tissue distribution, expression regulation, target genes, and physiological roles. For examples, HIF-1α seems to be expressed in nearly all cell types, whereas the expression of HIF-2α is more restricted and particularly abundant in some tissues such as blood vessels, kidney, lung, and colonic epithelia as well as in certain cell types such as astrocytes, chondrocytes, hepatocyes, macrophages, and adipocytes. Also, it has been recently demonstrated that expression and activity of HIF-1α and HIF-2α are selectively regulated at the

Figure 3. Oxygen deprivation leads to accumulation and activation of HIFα. In normoxia, sufficient oxygen supply ensures hydroxylation of proline and asparagine residues by prolyl hydroxylase domain-containing proteins (PHDs) and factor inhibiting HIF (FIH), respectively. Hydroxylated proline promotes to recruitment of the von Hippel-Lindau (VHL) E3 ubiquitin ligase, which leads to degradation of HIFα subunits. In addition, Hydroxylated asparagine promotes to recruitment of factor inhibiting HIF (FIH), which is involved in inactivation of HIFα subunits by inhibiting interaction of HIFα with transcriptional co-activator, p300. However, in hypoxic condition, oxygen deprivation leads to inactivation of PHDs and FIH. Inactivation of hydroxylases results in stabilization or activation of HIFα dimerized with HIF-1β. bHLH–PAS, basic helix–loop–helix–PAS protein domain.


Normoxia
Active PHDs
Active FIH
OH
OH
OH
Pro
Pro
Asn
bHLH-PAS
HIFα
VHL
p300
Proteasomal degradation
Transcriptional repression
Hypoxia
inactive PHDs
Inactive FIH
HIFβ
Transcriptional activation

levels of transcription, translation, post-translational modification and protein stability (Patel and Simon, 2008). Mammalian silent information regulator 2 homolog (SIRT1), redox-senstive and NAD^+-dependent deacetylases, deacetylates lysine residues in N-terminal of HIF-1α, leading to inhibition of transcriptional activity of HIF-1α (Cho and Lumeng, 2011; Dioum et al., 2009). In contrast, deacetylation of HIF-2α by SIRT1 results in transcriptional activation of HIF-2α (Lim et al., 2010). Furthermore, HIF-1α and HIF-2α have their own physiological roles via induction of unique target genes, probably, through interaction with different co-factors. HIF-1α induces the expression of glycolytic enzymes such as phosphoglycerate kinase (PGK), lactate dehydrogenase (LDH-A), and carbonic anhydrase-9 (CAIX), while HIF-2α regulates expression of different genes including erythropoietin (EPO), octamer-binding transcription factor 4 (OCT4), transforming growth factor α (TGFα) and delta like ligand 4 (DLL4). In particular, the studies on HIF-1α and HIF-2α in solid tumors have revealed their opposing roles in cell growth, energy metabolism, NO homeostasis, and others. (An et al., 1998; Bertout et al., 2009; Gordan et al., 2007; Keith et al., 2012; Koshiji et al., 2004). For example, HIF-2α promotes cell proliferation by enhancing oncogene c-Myc activity in hypoxic tumor growth, whereas HIF-1α induces cell cycle arrest by inhibition of c-Myc activity (Gordan et al., 2007; Koshiji et al., 2004). Also, HIF-1α or HIF-2α knockout mice show embryonic lethality with different causes (Iyer et al., 1998; Tian et al., 1998). For instance, HIF-1α knockout mice are lethal due to defects in vascularization, whereas HIF-2α knockout mice are lethal due to defects

of bradycardia and reduced catecholamine. Thus, it has been prospected that HIF-1α and HIF-2α would play nonredundant roles in development as well as physiologic and pathologic conditions.

3) Adipose tissue and HIFα

All isoforms of HIFα and binding partner HIF-1β are known to be expressed in adipose tissue (Hatanaka et al., 2009; Shimba et al., 2004; Ye, 2009). However, to data, most studies for adipose tissue HIFs have been focused on HIF-1α in the aspect of adipose tissue inflammation (Halberg et al., 2009; Jiang et al., 2013; Regazzetti et al., 2009; Sun et al., 2011; Wood et al., 2009; Ye, 2009). Given that the expression of HIF-1α is induced in obese adipose tissue, several genetically modified models for adipocyte HIF-1α have been developed (Halberg et al., 2009; Jiang et al., 2013; Ye et al., 2007). In mice, overexpression of constitutively active HIF-1α in adipose tissue induces adipose tissue inflammation, fibrosis, and insulin resistance (Halberg et al., 2009; Jiang et al., 2013; Ye et al., 2007). On the contrary, adipose tissue specific HIF-1α knock-out mice reveal that they have smaller fat mass, macrophage infiltration, and fibrosis (Halberg et al., 2009; Jiang et al., 2013; Ye et al., 2007). In addition, studies on angiogenic function of HIFα family in adipose tissue have been also reported (Ambrosini et al., 2002; Geiger et al., 2011; Grosfeld et al., 2002; He et al., 2011; Zhang et al., 2010). Several groups have suggested that HIF-1α is crucial in regulation of pro-angiogenic factors including VEGFα in adipocytes (Ambrosini et al., 2002; Grosfeld et al., 2002; He et al., 2011;

Zhang et al., 2010). In addition, adipocyte-specific dominant negative HIF-1α expression results in reduction of angiogenesis, mitochondrial biogenesis, and thermogenesis in brown adipose tissue (Zhang et al., 2010). However, the functional roles of other isoforms, HIF-2α and HIF-3α, regarding of angiogenesis or inflammation in adipose tissue, have not been clearly elucidated yet.

4. Purposes

In the progress of obesity, adipose tissue undergoes rapid and dynamic changes in its size, structure, and components in response to excessive nutrients. Proper adipose tissue remodeling, accompanied by effective angiogenic and adipogenic responses, would be crucial for healthy adipose tissue expansion to resist metabolic stresses such as hypoxia and inflammation, whereas limited angiogenesis and rapid enlargement of adipocytes often result in pathologic adipose tissue expansion. Recent studies have suggested that adipose tissue hypoxia and inflammation are strongly involved in pathological expansion of adipose tissue, which is mediated by activated HIF-1α in many ways. Although the physiological and pathological role of HIF-1α in adipose tissue has been quite well-established, the functional roles of HIF-2α in adipose tissue are poorly understood. Emerging evidence for the roles of HIF-2α in other tissues proposes that HIF-2α may have not only common roles of with HIF-1α such as angiogenesis but also its own functions.

In this study, I have investigated the function of HIF-2α in adipocytes and macrophages composing adipose tissue and the effects on cross-talk between

adipocytes and macrophages. To gain further insights, I have examined *in vivo* roles of HIF-2α in obese adipose tissue using HIF-2α heterozygote mice. In chapter one, I have demonstrated that accumulated HIF-2α in adipocytes results in induction of gene expression for pro-angiogenic factors as well as recruitment of macrophages that have the ability to supply additional pro-angiogenic factors. Particularly, the increase of HIF-2α expression in hypertrophic adipocytes from short-term HFD-fed mice led me to suspect that adipocyte HIF-2α could be involved in adipose tissue remodeling to keep energy homeostasis in early stage of obesity. In chapter two, I have shown that HIF-2α attenuated pro-inflammatory property via induction of ARG1 in macrophages, which would prevent pro-inflammatory responses and insulin resistance in adipocytes. Consistently, haploid deficiency of HIF-2α in mice exacerbated adipose tissue inflammation and insulin resistance in HFD-fed obesity. Taken together, I suggest that HIF-2α would alleviate insulin resistance in obese adipose tissue through stimulation of angiogenesis against hypoxia and suppression of pro-inflammatory responses of ATMs induced by metabolic stresses. Especially, in contrast to HIF-1α, the anti-inflammatory effect of HIF-2α in ATMs was newly proposed as resolving mechanism of adipose tissue inflammation.

CHAPTER ONE:

Adipocyte hypoxia-inducible factor 2α promotes expression of pro-angiogenic factors and recruitment of macrophages for adipose tissue angiogenesis

Abstract

Excess caloric intake leads to rapid expansion of adipose tissue, which induces adipose tissue hypoxia because of the relative insufficiency of the vascular network. Angiogenesis to counteract hypoxia in adipose tissue is involved in the activation of hypoxia inducible factor (HIF) family to play essential roles in hypoxic responses, including vascular remodeling. However the functional role of HIF-2α is adipose tissue angiogenesis is poorly understood. In this study, I have demonstrated that activation of HIF-2α in adipocytes could increase the expression levels of pro-angiogenic factors as well as recruit macrophages for angiogenesis. In adipocytes, the overexpression of HIF-2α significantly increased the expression levels of pro-angiogenic genes such as VEGFα, ANGPTL4, ADM, and MMPs regardless of hypoxic condition. HIF-2α-overexpressing adipocytes also promoted the recruitment and migration of macrophages, which was mediated by nitric oxide signaling. Interestingly, macrophages co-cultured with adipocytes exhibited increased the expression levels of pro-angiogenic factors such as IGF-1 and PDGFβ. Furthermore, macrophages sensitively and drastically induced the expression of VEGFα and AMD mRNA more than adipocytes in chronic hypoxic condition, implying that macrophage recruited by adipocyte HIF-2α could participate in adipose tissue angiogenesis. Taken together, these data suggest that adipocytes HIF-2α might regulate adipose tissue remodeling to respond to metabolic changes via promoting expression of angiogenic genes and macrophage recruitment.

Introduction

Throughout animal evolution, adipose tissue has been implicated in a key storage organ to stock up on surplus nutrients as well as an endocrine organ to secrete adipokines such as leptin, adiponectin, and inflammatory cytokines. In health adipose tissue, a well-developed vascular network serves to transport nutrients and adipokines for the proper functional performance of adipose tissues (Cao, 2007; Hausman and Richardson, 2004; Rutkowski et al., 2009; Yilmaz and Hotamisligil, 2013). When nutrients are over-loaded, the size and growth of adipose tissue are sensitively and dynamically changed to become hypertrophic and/or hyperplasic adipose tissues. In obesity, enlarged adipocytes with limited angiogenesis lead to adipose tissue hypoxia, which cause local pro-inflammatory responses and insulin resistance in adipose tissue (Lee et al., 2011b; Sun et al., 2011). Therefore, during adipose tissue expansion, the maintenance of vascular homeostasis via angiogenesis in adipose tissue determines the patho-physiology of adipose tissue (Sun et al., 2011; Yilmaz and Hotamisligil, 2013).

In adipose tissue, adipocytes are a major cell population to produce various kinds of pro-angiogenic factors including VEGFα, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), transforming growth factor β (TGFβ), angiopoietin, and leptin (Cao, 2007; Hausman and Richardson, 2004). Among these, VEGFα has been suggested to be a key molecule in the regulation of adipose tissue angiogenesis from the studies using genetically modified mouse models of VEGFα in adipose tissue (Elias et al., 2012; Sun et al.,

2012; Sung et al., 2013). Ablation of vascular endothelial growth factor α (VEGFα) in adipocytes reduces adipose tissue vasculature, which results in functional defects of adipose tissue upon high-fat diet (HFD), accompanied with adipose tissue hypoxia, inflammation, and apoptosis (Sung et al., 2013). On the contrary, enhanced adipose tissue vasculature by overexpression of adipocyte VEGFα protects adipocytes against metabolic stresses and thus prevents the development of systemic insulin resistance in obese mice (Elias et al., 2012; Sun et al., 2012; Sung et al., 2013). Non-adipocyte cells such as adipose stromal cells, preadipocytes, and macrophages are also considered as a source of angiogenic factors (Cao, 2007; Hausman and Richardson, 2004). The recruitment of macrophages into adipose tissue is involved in the formation of vascular network for growing adipose tissue (Cho et al., 2007; Xu et al., 2012). In addition, high levels of inflammatory cytokines produced from adipose tissue macrophages are also expected to act as potent angiogenic factors (Cao, 2007; Hausman and Richardson, 2004). However, the angiogenic function of adipose tissue macrophages (ATMs) has not been clearly understood yet.

In the progress of obesity, hypoxia is an important factor to stimulate angiogenesis in adipose tissue. Rapidly enlarged adipocytes suffer from oxygen debt resulted from the imbalance between the supply and consumption of oxygen because of the relative insufficiency of the vascular network, which induces local hypoxia in adipose tissue (Hosogai et al., 2007; Sun et al., 2011; Wood et al., 2009; Ye et al., 2007). Recently, most hypoxic responses including the regulation of pro-

angiogenic factors such as VEGFα, adrenomedullin (ADM) and plasminogen activator inhibitor-1 (PAI-1) have been investigated with two hypoxia-inducible factors, HIF-1α and HIF-2α in adipocytes (Ambrosini et al., 2002; Geiger et al., 2011; Grosfeld et al., 2002; He et al., 2011; Ratcliffe, 2007; Zhang et al., 2010). The activities of both HIF-1α and HIF-2α are primarily controlled through oxygen-dependent post-translational stabilization, and these transcription factors commonly activate HRE-dependent gene expression (Keith et al., 2012). Nevertheless, differential regulation of HIF-1α and HIF-2α transcripts and their differential post-translational modifications contribute to the functional specificity of each HIF isoforms (Keith et al., 2012; Lofstedt et al., 2007). For example, a transcript level of HIF-2α appears to be more tissue selective than that of HIF-1α (Ema et al., 1997; Wiesener et al., 2003). Also, HIF-2α protein can be easily stabilized at relatively high oxygen tensions (about 5% O_2) than HIF-1α in some cell types because HIF-2α can escape from degradation at near-normoxic conditions via low efficiency of post-translational modification-mediated degradation or inactivation (Bracken et al., 2006; Holmquist-Mengelbier et al., 2006; Koivunen et al., 2004; Li et al., 2009; Lofstedt et al., 2007; Nilsson et al., 2005; Yan et al., 2007; Yang et al., 2010). Thus, the possibility that hypoxic phenotypes are exhibited by active HIF-2α at near-physiological condition has been suggested. Considering the distinctive regulation of HIF-2α, HIF-2α is proposed to be involved in adipose tissue angiogenesis during normal growth or the pathological expansion of adipose tissue in the early stage of obesity even without severe hypoxia.

Recent studies for hypoxic responses in adipose tissue have been focused on the inflammatory changes in HIF-1α-dependent or independent mechanisms (Hosogai et al., 2007; Sun et al., 2011; Wang et al., 2007; Wood et al., 2009; Ye et al., 2007). The *in vivo* studies form the modulation of adipocyte HIF-1α using genetic modification or pharmacological agents have proposed that the activation of HIF-1α is associated with chronic inflammation, fibrosis, and insulin resistance in adipose tissue (Halberg et al., 2009; Jiang et al., 2013; Sun et al., 2013). However, although the role of HIF-1α in path-physiologic condition is quite well-established, the role of adipocyte HIF-2α is poorly understood. Recently, HIF-2α has been proposed to be involved in the regulation of adipogenesis (Shimba et al., 2004; Wada et al., 2006). Despite of these, the angiogenic function of adipocyte HIF-2α in adipose tissue is largely unknown.

In this study, I demonstrate that HIF-2α activation leads to the induction of pro-angiogenic factors in adipocytes as well as the recruitment of macrophages that have the ability to supply additional pro-angiogenic factors. Considering the rapid induction of adipocyte HIF-2α upon short-term HFD, it is possible to suggest that adipocyte HIF-2α might take an active part in adipose tissue remodeling through the modulation of expression of angiogenic genes and macrophage recruitment in the early stage of obesity.

Materials and Methods

Animals and treatment. C57/BL6 mice were purchased from Central Lab Animal Inc. They were maintained under pathogen-free conditions, and were housed in solid-bottom cages with wood shavings for bedding in a room maintained at 25°C with a 12:12 hr light: dark cycle (lights on at 07:00). For short-term HFD, 8 weeks-old mice were fed HFD (60% of calories derived from fat; Research Diets Inc., New Brunswick, NJ) for 3 days, 1 week, and 3 weeks. All mice were euthanized, and dissected tissue specimens were immediately stored at -80°C until analysis. All animal procedures were in accordance with the research guidelines of the Seoul National University Animal Experiment Ethics Committee.

Adipose tissue fractionation. Fractionation of adipose tissue was performed as previously described, with minor modifications (Park et al., 2006). Briefly, epididymal adipose tissues (EATs) were digested in type I collagenase buffer and filtered through nylon mesh. After centrifugation, floating adipocytes fraction and pelleted stromal vascular cells (SVCs) fraction were washed several times and collected each other for RNA extraction.

Whole-mount immunofluorescence. Whole-mount immunofluorescence was performed as previously described, with minor modifications (Huh et al., 2013). For whole-mount immunofluorescence, EATs were dissected from normal chow diet (ND)- or HFD-fed mice for 1 week. Dissected adipose tissues were fixed with 1% paraformaldehyde and blocked with 5% goat serum in PBST for 1 hr. For whole-

mounted EAT were incubated overnight at 4°C with primary antibody (HIF-2α, 1:1000, Novus, CO; perilipin, 1:1,000, Fitzgerald, MA; CD11b 1:1000, eBioScience, CA; CD31, 1:1000, Millipore, MA). After being washed for 1 hr, samples were incubated with fluorescence-labeled secondary antibody for 4 hrs at room temperature then washed. Following staining with 4',6-diamidino-2-phenylindole (DAPI) (Vector Lab., CA), samples were observed using a Zeiss LSM510NLO confocal microscope. For detection of blood vessel, in adipose tissue stained with CD31 antibody, images obtained via z-section in 80 μm thickness were accumulated using Zeiss LSM Image Browser software.

3T3-L1 adipocyte differentiation. 3T3-L1 preadipocytes were grown to confluence in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT) supplemented with 10% bovine calf serum (HyClone). At 2 days after confluence, differentiation of the 3T3-L1 cells were stimulated with DMEM containing 10% fetal bovine serum (FBS; HyClone), methylisobutylxanthine (500 μmol/l), dexamethasone (1 μmol/l), and insulin (5 μg/ml) for 2 days. Then, culture medium was changed with DMEM containing 10% FBS and insulin (5 μg/ml). For induction of hypertrophic adipocytes, differentiated adipocytes were cultured with free-fatty acid mixture (each 0.25mM palmitic acid, stearic acid, and oleic acid) for 7 days as previously described, with minor modifications (Yeop Han et al., 2010).

Isolation of peritoneal macrophage. 8 week-old Mice were injected intraperitoneally with sterile fluid thioglycollate solution (2 ml per mouse). After 3

days, the peritoneal cells were harvested by washing the peritoneal cavity with PBS containing 5 mM EDTA. Primary peritoneal macrophages were cultured with DMEM with 10% FBS to allow cell adherence. The non-adherent cells were removed by washing.

Adenovirus infection. HIF-2α adenovirus was generously provided by Jang-Soo Chun (Yang et al., 2010). As a negative control, GFP adenovirus (Neurogenex, South Korea) was used. Fully differentiated 3T3-L1 adipocytes were incubated with serum-free DMEM and 100 MOI of adenovirus for 16 hrs. Then, the culture medium was replaced with fresh medium. Each experiment was performed at 48 hrs after viral infection.

Chemotaxis assay. Differentiated 3T3-L1 adipocytes were infected with Ad-GFP or Ad-HIF-2α. After 2days, adipocytes were co-cultured with THP-1 monocytes (5 X 10^4 cells / ml), which were pre-stained with Cell-tracker (Sigma-Aldrich, MO), with or without 0.5 mM L-NAME, iNOS inhibitor (Sigma-Aldrich). 10 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich) was used as positive control. After 24 hrs, nonadherent THP-1 cells were removed by washing with PBS. THP-1 cells adhered to 3T3-L1 adipocytes were fixed with 3.7% paraformaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Lab., CA). Samples were observed using a Zeiss LSM510NLO confocal microscope. Cell-tracker-positive cells were quantitated using imaging analysis program (Zeiss LSM Image Browser). For chemotaxis assay, THP-1 cells were maintained with RPMI containing 10% FBS.

Migration assay. Ad-GFP- or ad-HIF-2α-infected 3T3-L1 adipocytes were incubated in DMEM containing 10% with or without 0.5 mM L-NAME for 2 days and then the medium were switched to serum-free DMEM medium for 24 hrs. Thereafter, conditional medium were collected from the cells. Primary cultured peritoneal macrophages (5 X 10^5 cells / ml) were seeded on trans-well with a pore size of 8.0 μm. The next day, peritoneal macrophages were incubated in conditional medium and treated of 10 ng/ml LPS as positive control. After 6 hrs or 24 hrs, macrophages on the top of the trans-well were wiped out using a cotton swab, and fixed, then stained with DAPI. The migrating cells were observed using a Zeiss LSM510NLO confocal microscope, quantitated using imaging analysis program (Zeiss LSM Image Browser).

Insulin-stimulated glucose uptake assay. Insulin stimulated glucose uptake in 3T3-L1 adipocytes was determined by measuring [^{14}C]2-deoxy-glucose uptake as previously described, with minor modifications (Jeong et al., 2009). Briefly, Ad-GFP- or Ad-HIF-2α-infected 3T3-L1 adipocytes were incubated in DMEM containing 10% FBS with or without L-NAME for 2 days and the medium were switched to low-glucose DMEM containing 0.1% BSA for 8 hrs. The cells were stimulated with or without 50 nM insulin for 15 min. Glucose uptake was initiated by the addition of the final concentration of [^{14}C]2-deoxy-D-glucose (PerkinElmer Life, MA) for in HEPES buffer saline for 15 min. The reaction was terminated by several times of cold-PBS washing. After the cells were lysed with 0.1% SDS, ^{14}C radioactivity was measured using scintillation counter. Measured ^{14}C radioactivities

were normalized by total protein concentration of whole cell lysates.

Quantitative RT-PCR. Total RNA was isolated from 3T3-L1 adipocytes, peritoneal macrophages or EAT as described previously (Choe et al., 2007). cDNA was synthesized using the M-MuLV reverse transcriptase kit according to the manufacturers' protocol (Thermo Fisher Scientific, MA). The primers used for quantitative real-time PCR (Bioneer, South Korea) and their sequences are provided in supplementary Table I.

Western blot analysis. Western blot analysis was performed as described previously (Choe et al., 2007). 3T3-L1 adipocytes or EATs were lysed with NETN buffer. The proteins were separated on SDS-PAGE gels and transferred to PVDF membranes (Millipore). Blots were blocked with 5% nonfat milk and probed with primary antibodies, HIF-2α (Novus), HIF-1α and LaminB (Abcam, MA), iNOS (Santa Cruz Biotechnology, CA), and β-actin (Sigma-Aldrich). The blots were visualized with horseradish peroxidase (HRP)–conjugated secondary antibodies (Sigma-Aldrich) and enhanced chemiluminescence (ECL). For the detection of MAPK activation in macrophages, peritoneal macrophages were lysed after conditioned medium incubation for 1 hr using antibodies against pERK (Thr980; Cell Signaling, MA), p-p38 (Thr180/Tyr182; BD bioscience, CA), ERK and p38 (Santa Cruz Biotechnology). For detection of insulin signaling in 3T3-L1 adipocytes, Ad-GFP- or Ad-HIF-2α- 3T3-L1 cells were incubated in DMEM containing 10% FBS for 2 days and the medium were switched to low-glucose

Table 1. qRT-PCR primer sequences

Gene	*5′sequence*	*3′sequence*
ACC1	GAGTGACTGCCGAAACATCT	GCCTCTTCCTGACAAACGAG
ACO1	TGAAAACAGTTGTGCCTTGC	ACAGAGCCATGAGCGAGAGT
Adiponectin	GGCAGGAAAGGAGAGCCTGG	GCCTTGTCCTTCTTGAAGAG
AMD	TCAGAGCATCGCCACAGAAAT	TAGCTGCTGGATGCTTGTAG
Angptl4	GGCTGGTGGTTTGGTACCTGT	CCGTTGCCGTGGGATAGA
ATGL	CTCCAGCGGCAGAGTATAGG	ACCATCACAGTGTCCCCATT
CD36	GAGCAACTGGTGGATGGTTT	GCAGAATCAAGGGAGAGCAC
CPT1α	ACTCCTGGAAGAAGAAGTTC	GTATCTTTGACAGCTGGGAC
Cyclophilin	CAGACGCCACTGTCGCTTT	TGTCTTTGGAACTTTGTCTG
FAS	CGGTAGCTCTGGGTGTA	TGCTCCCAGCTGCAGGC
FGF-2	CACCAGGCCACTTCAAGGA	GATGGATGCGCAGGAAGAA
GLUT4	GATTCTGCTGCCCTTCTGTC	ATTGGACGCTCTCTCTCCAA
GPx	GGGCAAGGTGCTGCTCATTG	AGAGCGGGTGAGCCTTCTCA
HGF	TAGGAGCCACAAGGATCTGG	ACATGAAGCAGGAGGAGGTG
HIF-1α	CAAGATCTCGGCGAAGCAA	GGTGAGCCTCATAACAGAAGCTTT
HIF-2α	GGTTCGGGAGCACACTGTAT	CCTTCCTTCACAGAGCCAAG
HSL	GGAGCACTACAAACGCAACG	TCGGCCACCGGTAAAGAG
IGF-1	TGCTCTTCAGTTCGTGTG	ACATCTCCAGTCTCCTCAG
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
IL-1β	TGCAGAGTTCCCCAACTGGTACATC	GTGCTGCCTAATGTCCCCTTGAATC
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
iNOS	AATCTTGGAGCGAGTTGTGG	CAGGAAGTAGGTGAGGGCTT
IR	CGAGTGCCCGTCTGGCTATA	GGCAGGGTCCCAGACATG
IRS2	GGAGAACCCAGACCCTAAGC	GATGCCTTTGAGGCCTTCAC
LDLR	AGGCTGTGGGCTCCATAGG	TGCGGTCCAGGGTCATCT
LPL	GCCCAGCAACATTATCCAGT	GGTCAGACTTCCTGCTACGC
mCAD	AGGTTTCAAGATCGCAATGG	CTCCTTGGTGCTCCACTAGC
MGL1	ATGATGTCTGCCAGAGAACC	ATCACAGATTTCAGCAACCTTA
MGL2	TTAGCCAATGTGCTTAGCTG	GGCCTCCAATTCTTCTTGAAACCT
MMP-13	TGATGGACCTTCTGGTCTTCTGG	CATCCACATGGTTGGGAAGTTCT

MMP-3	TCCTGATGTTGGTGGCTTCAG	TGTCTTGGCAAATCCGGTGTA
MMP-9	ACCACATCGAACTTCGA	CGACCATACAGATACTG
MRC1	TACAGCTCCACGCTATGGATT	CACTCTCCCAGTTGAGGTACT
NOX2	TTGGGTCAGCACTGGCTCTG	TGGCGGTGTGCAGTGCTATC
NOX4	TTGCCTGGAAGAACCCAAGT	TCCGCACAATAAAGGCACAA
p22phox	GTCCACCATGGAGCGATGTG	CAATGGCCAAGCAGACGGTG
p47phox	GATGTTCCCCATTGAGGCCG	GTTTCAGGTCATCAGGCCGC
p67phox	CTGGCTGAGGCCATCAGACT	AGGCCACTGCAGAGTGCTTG
PDGFβ	CTGAGCTGGACTTGAACATG	TTAAACTTTCGGTGCTTGCC
PGC1α	CCTCCTCATAAAGCCAACCA	GGGCCGTTTAGTCTTCCTTT
PPARγ	GAACGTGAAGCCCATCGAGG	GTCCTTGTAGATCTCCTGGA
Pref-1	TCTGCGAAATAGACGTTCGGGCTTG	GCCATCGTTCTCGCATGGGTTAGGG
SAA	AGCGATGCCAGAGAGGCTGT	ACCCAGTAGTTGCTCCTCTT
SCD1	TGGGTTGGCTGCTTGTG	GCGTGGGCAGGATGAAG
SOD2	CATGTCTGTGGGAGTCCAAGGTTCA	GTCAATCCCCAGCAGCGGAATAAG
SREBP1c	CTGGACCACAGAAAGGTGGA	GGACTGAAGCTGGTGACTGC
TGFβ	AAGTTGGCATGGTAGCCCTT	GCCCTGGATACCAACTATTGC
TNFα	CGGAGTCCGGGCAGGT	GCTGGGTAGAGAATGGATCA
VEGFα	GGAGATCCTTCGAGGAGCACTT	GGCGATTTAGCAGCAGATATAAGAA
VLDLR	TGACGCAGACTGTTCAGACC	GCCGTGGATACAGCTACCAT

DMEM containing 0.1% BSA for 8 hrs. Cells were treated with 50nM insulin for 30 min and then lysed for subsequent analysis. For detection of insulin signaling cascades, antibodies against pAkt (Ser473), Akt, and pGSK (Ser9) (Cell Signaling, MA) and GSK (BD bioscience) were used.

Measurement of nitric oxide level and ROS level. Nitrite was measured using the Griess reaction as described previously (Park et al., 2006). Cultured media (100 μl) were collected and incubated with an equal volume of Griess reagent (Sigma-Aldrich) for 10 min at room temperature. The nitrite concentration was determined by the absorbance at 550 nm, using sodium nitrite as a standard. Cellular ROS was measured using chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (chloromethyl-H_2DCF-DA; Invitrogen, NY). Adipocytes were incubated with DCF-DA in dark for 20 min and washed with PBS. The fluorescence of DCF-DA was monitored by a Zeiss LSM510NLO confocal microscope. 100 mM H_2O_2 was used as a positive control of ROS measurement.

RNA preparation and microarray analysis. For microarray analysis, total RNA was isolated from EAT of ND-fed mice or short-term HFD-fed mice for 3 days and 1 week. RNA quality was assessed by running electrophoresis in a denaturing agarose gel. Ten micrograms of purified total RNA was prepared according to Affymetrix protocols. Prepared cRNA was hybridized to Affymetrix Gene Chip Mouse Gene 1.0 ST Array. Microarray data analyzed using Affymetrix Gene Chip Operating Software and Gene Set Enrichment Analysis (GSEA) software.

Measurement of cellular triglyceride levels The cellular contents of triglycerides in adipocytes cells were measured using triglycerides assay kit (Thermo Fisher Scientific). Analysis was performed according to the manufacturers' protocol.

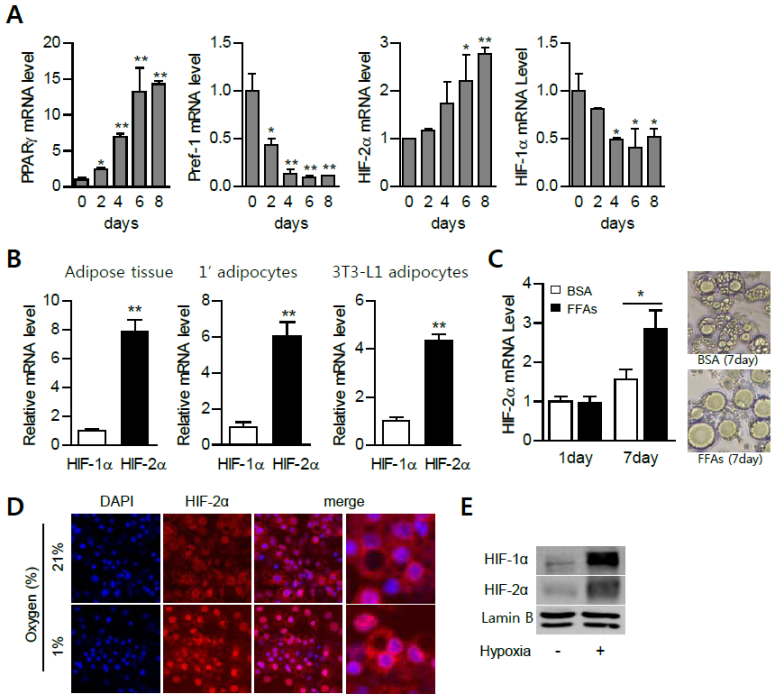
Statistical analysis. Results represent data from at least more than two times independent experiments. Error bars represent standard deviation, and *P* values are calculated from the Student's t-test or two-way ANOVA.

Results

Adipocyte HIF-2α is induced in adipogenic, hypertrophic, and hypoxic condition

In adipocytes, HIFα family has been suggested to play a central role in the regulation of hypoxia-response gene expression under hypoxic condition (Geiger et al., 2011). However, the regulation of HIF-2α in adipocytes has not been thoroughly investigated. I first compared the mRNA level of HIF-2α with that of HIF-1α in differentiated 3T3-L1 adipocytes. The expression level of peroxisome proliferator-activated receptor γ (PPARγ) mRNA, as the key adipocyte differentiation marker gene, was highly induced, whereas that of preadipocyte factor-1 (Pref-1), as a preadipocyte marker gene, was decreased for adipogenesis (Fig. 4A). Consistent with the previous finding that the expression level of HIF-2α mRNA is increased during adipogenesis (Shimba et al., 2004), HIF-2α mRNA was gradually elevated in the process of adipocyte differentiation, and the mRNA level of HIF-2α was higher than that of HIF-1α in fully differentiated adipocytes (Fig. 4A and B). Also, HIF-2α mRNA was abundantly expressed in adipose tissues and isolated primary adipocytes (Fig. 4B). In hypertrophic adipocytes treated with free fatty acids (FFAs) mixture for 7 days (Yeop Han et al., 2010), the expression level of HIF-2α mRNA was increased, whereas HIF-2α mRNA was not changed by acute treatment of FFAs mixture for 1 day (Fig. 4C). Besides, adipocyte HIF-2α protein was accumulated in the nuclei of adipocytes in response to hypoxia (Fig. 4D and E). These results

Figure 4. Adipocyte HIF-2α is increased by adipogenic, hypertrophic, and hypoxic stimulation. A: Expression level of HIF-2α, HIF-1α, PPARγ (adipocyte marker), and Pref-1 (preadipocyte marker) mRNA during adipose differentiation in 3T3-L1 cells was determined by qRT-PCR. Data represent mean ± SD. **P* < 0.05, and ***P* < 0.05 vs. 0 day preadipocyte control by Student's *t*-test. B: Expression level of HIF-2α was compared with HIF-1α in epididymal adipose tissues (EATs), and primary cultured mouse adipocytes, and matured 3T3-L1 adipocytes. Data represent mean ± SD. **P* < 0.05, and ***P* < 0.05 vs. vs. HIF-1α mRNA level by Student's *t*-test. C: To induce hypertrophic adipocyte, 3T3-L1 adipocytes were differentiated for 6day then incubated with FFAs mixture or BSA control for additional 7 days; image of control adipocyte (up) and hypertrophic adipocytes (bottom). Expression level of HIF-2α mRNA was determined by qRT-PCR at 1 and 7 day after incubation of FFAs mixture. Data represent mean ± SD. **P* < 0.05 vs. BSA control by Student's *t*-test. D-E: Protein level of HIF-2α in adipocytes under normoxic or hypoxic (1% O_2) was detected using immunocytofluorescence for the nucleus (blue), and HIF-2α (red) (D). Nuclear extracts from these adipocytes were subjected to Western blotting analysis with antibodies against HIF-1α, HIF-2α, and Lamin B. Lamin B was used as the loading control (E). All mRNA expression levels were normalized by the level of 39yclophilin mRNA.


A
PPARγ mRNA level
Pref-1 mRNA level
HIF-2α mRNA level
HIF-1α mRNA Level
0 2 4 6 8
days
B
Adipose tissue
1′ adipocytes
3T3-L1 adipocytes
Relative mRNA level
HIF-1α HIF-2α
C
BSA
FFAs
HIF-2α mRNA Level
1day
7day
BSA (7day)
FFAs (7day)
D
DAPI
HIF-2α
merge
Oxygen (%)
21%
1%
E
HIF-1α
HIF-2α
Lamin B
Hypoxia - +

suggest the possibility that increased HIF-2α could function as a mediator of hypoxic phenotype in adipocytes in the progress of obesity.

HIF-2α is increased in adipocytes from short-term HFD-fed mice

Increase of HIF-2α mRNA in free-fatty acid-induced hypertrophic adipocytes led us to test whether the level of HIF-2α mRNA might be elevated in *in vivo* hypertrophic adipocytes. Consistent with previous report (Lee et al., 2011b), short-term HFD for 1 week induce hypertrophic adipocytes in epididymal adipose tissue, accompanied with increase of body weight and adipose tissue mass of mice (Fig. 5). In adipose tissue from short-term

HFD-fed mice, the mRNA levels of HIF-2α and its target gene such as VEGFα were significantly increased (Fig. 6A and B). To determine the expression level of HIF-2α mRNA in adipocytes, primary adipocytes were isolated from adipose tissue. Compared to normal chow diet (ND)-fed mice, the mRNA levels of HIF-2α and VEGFα in adipocytes were significantly promoted in HFD-fed mice (Fig. 6C). On the contrary, the mRNA level of adiponectin tended to decrease (Fig. 6C). Moreover, when whole-mount immunofluorescence was performed, HIF-2α-positive signals were enhanced in adipocytes of short-term HFD-fed mice (Fig. 6D), implying that adipocyte HIF-2α could be elevated in the early stage with 1 week HFD.

Markers of macrophage and endothelial cells are increased in adipose tissue of

Figure 5. Short-term HFD induces hypertrophic adipocytes accompanied with increase of body weight and adipose tissue mass. A-B: Eight-week-old mice (n = 5 per group) were fed ND or HFD for 1 week. Body weight (A) and the weights of tissues (B) were measured on the last day. Data represent mean ± SD. *$P < 0.05$, and **$P < 0.01$ vs. ND diet control by Student's *t*-test. C: To detect adipocyte size, whole-mount immunofluorescence for the nucleus (blue), and perilipin (green) was performed on their EATs

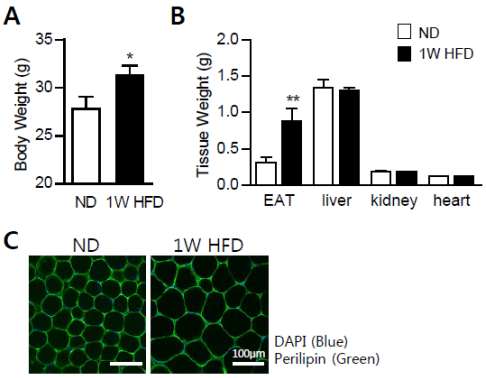
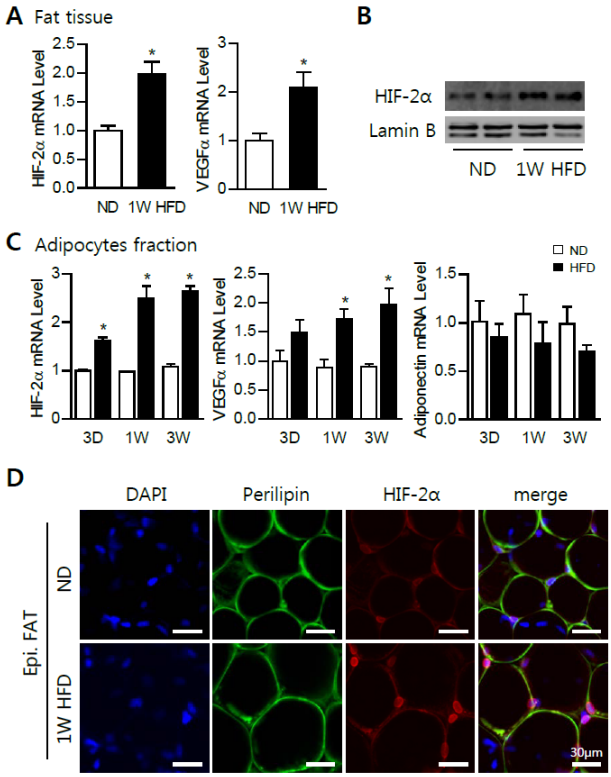

A
Body Weight (g)
35
30
25
20
*
ND
1W HFD
B
Tissue Weight (g)
2.0
1.5
1.0
0.5
0.0
**
ND
1W HFD
EAT
liver
kidney
heart
C
ND
1W HFD
100μm
DAPI (Blue)
Perilipin (Green)

Figure 6. Short-term HFD accumulates HIF-2α in adipocytes. A-B: Expression level of HIF-2α and VEGFα mRNA in EATs of ND- or HFD-fed mice (n=5 per group) for 1 week (A) and nuclear extracts from the same adipose tissue were subjected to Western blot analysis with antibodies specific for HIF-2α and Lamin B. Lamin B was used as the loading control (B). C: Expression level of HIF-2α, VEGFα, and adiponectin mRNA in adipocytes isolated form EATS of ND- or HFD-fed mice for 3 days, 1, or 3 weeks was determined by qRT-PCR. D: Expression pattern of HIF-2α in EATS was detected by whole-mount immunofluorescence analysis for the nucleus (blue), perilipin (green), and HIF-2α (red). All mRNA expression levels were normalized by the level of 44yclophilin mRNA. Data represent mean ± SD. *$P < 0.05$ vs. ND control by Student's *t*-test.


A Fat tissue
HIF-2α mRNA Level
VEGFα mRNA Level
ND 1W HFD
B
HIF-2α
Lamin B
ND 1W HFD
C Adipocytes fraction
ND
HFD
HIF-2α mRNA Level
VEGFα mRNA Level
Adiponectin mRNA Level
3D 1W 3W
D
DAPI
Perilipin
HIF-2α
merge
Epi. FAT
ND
1W HFD
30μm

short-term HFD-fed mice

To determine whether, adipose tissue remodeling was accompanied with the accumulation of adipocyte HIF-2α at early stage of HFD, whole-mount immunofluorescence analyses were performed with markers of adipocytes (periliphin), macrophages (CD11b), and endothelial cells (CD31). As shown in Figure 7A and B, blood vessel density was maintained although adipocyte size was increased more than 2 fold, and consequently vessel area per adipocyte was increased. In addition, the number of macrophages was increased upon short-term HFD and many of them were found to be located around the blood vessel (Fig. 7C).

To further investigate the structural change in adipose tissue upon short-term HFD, microarray analysis was performed with adipose tissue and liver from HFD-fed mice for 0, 3, and 7 days. Interestingly, the mRNA levels of macrophage markers such as CD68 and F4/80 were significantly elevated along with M2 markers such as macrophage galactose N-acetyl-galactosamine-specific lectin 1 (MGL1), MGL2, macrophage mannose receptor 1 (MRC1), MRC2, and CD163 in adipose tissue at only 3 days of HFD (Fig. 8A). Simultaneously, the mRNA levels of endothelial cell markers such as CD31, CD34, von willebrand factor (vWF), melanoma cell adhesion molecule (mCAM), cadherin 2 (CDH2), and cadherin 5 (CDH5) were also significantly increased in adipose tissue of short-term HFD-fed mice (Fig. 8A). These results indicate that macrophages and endothelial cells are actively and rapidly recruited to adipose tissue remodeling to respond for HFD. On the contrary, in liver, structural change was not exhibited and differences in mRNA

Figure 7. Short-term HFD increases vessel area faced with hypertrophic adipocytes. A-C: Whole-mount immunofluorescence analysis for blood vessel density and adipose tissue macrophage detection was performed on EATS form ND- and HFD-fed mice for 1 week. The nucleus (blue); Perilipin (green); CD11b (macrophage marker; Yellow). CD31 (endothelial cell marker; red) signal was accumulated 80 μm thickness (A). Blood vessel area, average adipocyte size, and blood vessel area per adipocyte was quantified with whole-mount immunofluorescence images (B). Data represent mean ± SD. *$P < 0.05$ vs. ND control by Student's *t*-test. Merged image of CD11b and CD31 signal (C).

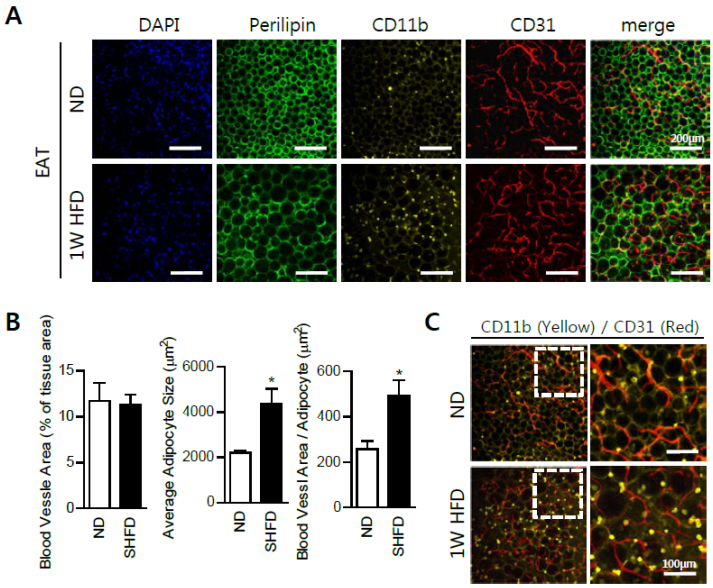
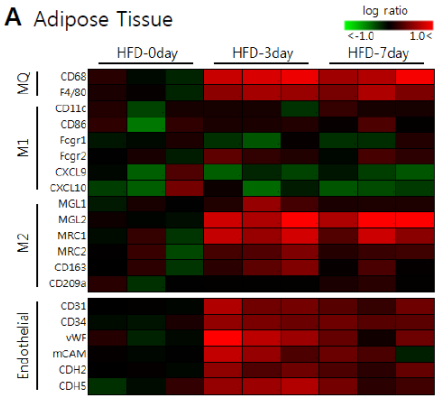
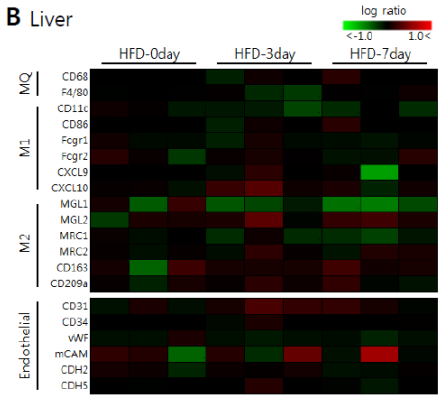

A
DAPI
Perilipin
CD11b
CD31
merge
EAT
ND
1W HFD
200μm
B
Blood Vessle Area (% of tissue area)
15
10
5
0
ND
SHFD
Average Adipocyte Size (μm²)
6000
4000
2000
0
*
ND
SHFD
Blood Vessl Area / Adipocyte (μm²)
600
400
200
0
*
ND
SHFD
C
CD11b (Yellow) / CD31 (Red)
ND
1W HFD
100μm

Figure 8. Short-term HFD increases levels of macrophage and endothelial cell markers in adipose tissue. A-B: A heat map that displays the relative Cy3/Cy5 ratios of RNAs expressed in adipose tissue (A) and liver (B) of HFD-fed mice for 0, 3, and 7 days. The mRNA level of indicated genes is displayed as higher (red) or lower (green) in adipose tissue and liver of HFD-fed mice for 3 and 7 days compared to 0 days. C-D: Gene-set enrichment analysis against the KEGG database for differentially enriched pathways in adipose tissue between ND-fed mice and HFD-fed mice for 7 days. (C) Top 10 list of significantly enriched pathway. (D) Enrichment plot for VEGF signaling pathway up-regulated in adipose tissue of HFD-fed mice for 7 days and higher ranked genes in the ranked list.



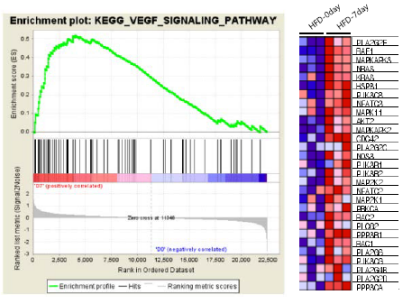


C

No.	Enriched KEGG Pathway	SIZE	NES	FDR q-val
1	PATHOGENIC_ESCHERICHIA_COLI_INFECTION	47	2.1996	0.0000
2	FC_EPSILON_RI_SIGNALING_PATHWAY	77	1.9080	0.0048
3	FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS	87	1.9112	0.0052
4	CELL_CYCLE	113	1.9212	0.0062
5	ECM_RECEPTOR_INTERACTION	79	1.9595	0.0063
6	GAP_JUNCTION	77	1.9312	0.0075
7	**VEGF_SIGNALING_PATHWAY**	71	1.8185	0.0093
8	B_CELL_RECEPTOR_SIGNALING_PATHWAY	72	1.8218	0.0100
9	HYPERTROPHIC_CARDIOMYOPATHY_HCM	82	1.8497	0.0104
10	NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY	97	1.8050	0.0105

* Top 10 of significantly enriched KEGG pathway
* KEGG - Kyoto encyclopedia of genes and genomes
* SIZE - Number of genes in pathway
* NES - Normalized enrichment score
* FDR - False discovery rate

D

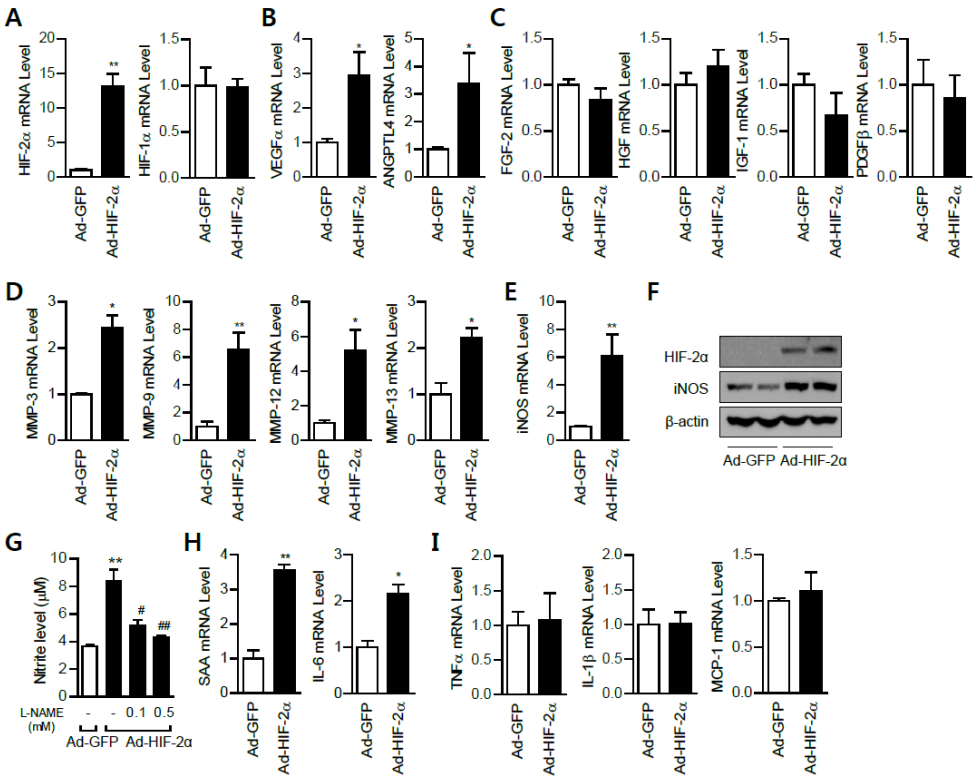


levels of macrophage and endothelial cell marker genes were not observed by short-term HFD (Fig. 8B). Furthermore, data from enrichment analysis showed significant increase of genes linked to VEGF signaling (Fig. 8C and D), which indicating that angiogenesis would be stimulated in adipose tissue of short-term HFD-fed mice to maintain homeostatic regulation of blood vessels. These correlative data suggest the possibility that adipocyte HIF-2α accumulation, macrophage recruitment, and angiogenesis are closely linked with each other.

In adipocytes, HIF-2α overexpression stimulates the expression levels of pro-angiogenic factors and nitric oxide production

To mimic the increase of HIF-2α in hypertrophic adipocytes without the effects of HIF-1α, HIF-2α was overexpressed in 3T3-L1 adipocytes using adenovirus and then mRNA levels of target genes were analyzed in normoxic condition (Fig. 9A). Interestingly, under normoxic condition, pro-angiogenic factors such as VEGFα and ANGPTL4 were significantly induced by HIF-2α overexpression whereas pro-angiogenic genes such as FGF-2, HGF, IGF-1, and platelet-derived growth factor β (PDGFβ) were not changed (Fig. 9B~C). In addition, adipocyte HIF-2α induced gene expression of tissue remodeling factors such as matrix metalloproteinases (MMPs) (Fig. 9D), implying that HIF-2α would be involved in the remodeling of extra cellular matrix (ECM) in adipose tissue upon HFD. Furthermore, HIF-2α-overexpressing adipocytes exhibited increased nitric oxide (NO) production via inducible nitric oxide synthase (iNOS or NOS2)

Figure 9. HIF-2α overexpression induces expression of pro-angiogenic factors, MMPs, and iNOS in adipocytes. A-H: 3T3-L1 adipocytes were infected with Ad-HIF-2α or Ad-GFP. After 48 hr, total RNA was isolated and analyzed by qRT-PCR for HIF-2α and HIF-1α (A), pro-angigogenic genes (VEGFα, ANGPTL4, FGF-2, HGF, IGF-1, and PDGFβ) (B-C), MMPs (MMP-3, MMP-9, MMP-12 and MMP-13) (D), and iNOS (E). Total lysates were subjected to western blot analysis using specific antibodies (HIF-2α, iNOS and β-actin). B-actin was used as the loading control. (F) Ad-GFP- or Ad-HIF-2α-infected adipocytes were treated with or without L-NAME, iNOS inhibitor (0.1 and 0.2 mM). After 48 hr, cultured media of these cells were used for measurement of NO concentration (G). Data represent mean ± SD. $*P < 0.05$, and $**P < 0.01$ vs. Ad-GFP control; $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ vs. Ad-HIF-2α control with no treatment by Student's *t*-test. mRNA level of inflammatory genes (SAA, IL6, TNFα, IL1β) was measured by qRT-PCR in the same samples (H-I). $P < 0.05$, and $**P < 0.01$ vs. Ad-GFP control by Student's *t*-test. All mRNA expression levels were normalized by the level of 52yclophilin mRNA.


A
HIF-2α mRNA Level
HIF-1α mRNA Level
B
VEGFα mRNA Level
ANGPTL4 mRNA Level
C
FGF-2 mRNA Level
HGF mRNA Level
IGF-1 mRNA Level
PDGFβ mRNA Level
Ad-GFP
Ad-HIF-2α
D
MMP-3 mRNA Level
MMP-9 mRNA Level
MMP-12 mRNA Level
MMP-13 mRNA Level
E
iNOS mRNA Level
F
HIF-2α
iNOS
β-actin
Ad-GFP Ad-HIF-2α
G
Nitrite level (μM)
L-NAME (mM)
- - 0.1 0.5
Ad-GFP Ad-HIF-2α
H
SAA mRNA Level
IL-6 mRNA Level
I
TNFα mRNA Level
IL-1β mRNA Level
MCP-1 mRNA Level

induction, which was abolished by treatment with NOS inhibitor, L-NAME (Fig. 9E~G). NO is a signaling molecule that has various effects on cellular processes depending on its concentration. High concentrations of NO contribute to the induction of insulin resistance in obesity whereas physiological level of NO (less than 10 nM) is involved in the stimulation of angiogenesis and blood flow (Schwentker et al., 2002; Singh and Agarwal, 2007). While HIF-2α overexpression in adipocytes increased the mRNA levels of several inflammatory mediators such as IL-6 and SAA (Fig. 9H), the expression levels of key pro-inflammatory cytokines such as TNFα, IL-1β, and MCP-1 that cause glucose intolerance in obesity were not changed (Fig. 9I), implying that the activation of HIF-2α appears to be selectively and partly involved in adipose tissue inflammation. Thus, these data indicate that accumulated HIF-2α in adipocytes could primarily contribute to the production of pro-angiogenic factors and tissue remodeling factors, which might promote angiogenesis for adipose tissue remodeling in response to obese condition.

Adipocyte HIF-2α overexpression promotes the recruitment of monocytes onto adipocytes

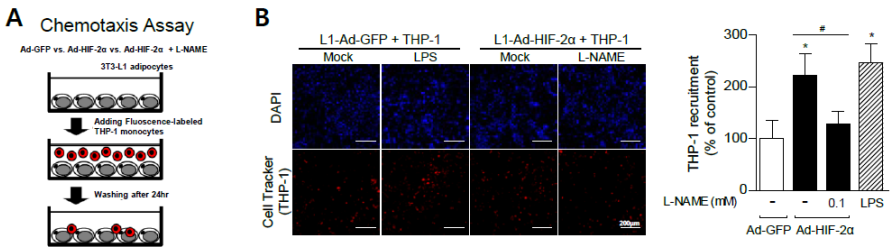
Infiltration of macrophage was observed in adipose tissue of short-term HFD-fed mice (Fig. 7 and 8). To confirm whether the activation of HIF-2α is also involved in this macrophage recruitment, the effects of adipocyte HIF-2α overexpression on the physical interaction between adipocytes and monocytes were examined using direct co-culture system. For chemotaxis assays, mock adenovirus

(Ad-Mock)- and HIF-2α adenovirus (Ad-HIF-2α)-infected 3T3-L1 adipocytes were incubated with Cell Tracker-labeled THP-1 monocyte cells as illustrated in figure 10A. Interestingly, more THP-1 cells were recruited to the HIF-2α-overexpressing adipocytes compared with the control adipocytes (Fig. 10B). These results suggest that adipocyte HIF-2α could affect the recruitment of circulating monocytes into adipose tissue. In addition, monocytes recruitment by adipocyte HIF-2α was considerably attenuated by NOS inhibitor, L-NAME, indicating that NO signaling would be involved in monocyte recruitment.

Adipocyte HIF-2α overexpression stimulates macrophage migration but does not affect macrophage polarity

Next, the effect of adipocyte HIF-2α overexpression on macrophage migration was investigated. For migration assay, macrophages were incubated on permeable trans-wells (8 μm pore size) with conditioned medium from the culture of adipocytes infected with Ad-HIF-2α or Ad-GFP control in the presence or absence of NOS inhibitor, L-NAME, as illustrated in figure 11A. As shown in figure 11B, certain mediators secreted from adipocytes activated macrophage MAP kinase, such as p38 and ERK, related to macrophage migration, which was enhanced by HIF-2α overexpression in adipocytes. With MAP kinase activation, conditioned medium from HIF-2α-overexpressing adipocytes significantly promoted macrophage migration compared to control medium (Fig. 11C). In addition, these effects were abolished in macrophages incubated with conditioned medium from

Figure 10. HIF-2α-overexpressing adipocytes exhibites increased recruitment of monocytes during direct co-culture. A: Illustration of chemotaxis assay with direct co-culture THP-1 monocyte with 3T3-L1 adipocytes. Monocyte were stained with Cell-Tracker then co-cultured with either Ad-GFP- or Ad-HIF-2α-infected adipocytes with or without L-NAME for 24 hr. Nonadherent monocytes were removed by washing. LPS was used as positive-control. B: Monocytes attached onto the adipocytes were observed using a Zeiss LSM510NLO confocal microscope. Quantitative measurements of Cell-Tracker-positive cells were obtained by Zeiss LSM Image Browser software and normalized by DAPI (B). Data represent mean ± SD. $^{*}P < 0.05$ vs. Ad-GFP control with no treatment; $^{\#}P < 0.05$ vs. Ad-HIF-2α control with no treatment by Student's *t*-test.


A
Chemotaxis Assay
Ad-GFP vs. Ad-HIF-2α vs. Ad-HIF-2α + L-NAME
3T3-L1 adipocytes
Adding Fluoscence-labeled THP-1 monocytes
Washing after 24hr
B
L1-Ad-GFP + THP-1
L1-Ad-HIF-2α + THP-1
Mock
LPS
Mock
L-NAME
DAPI
Cell Tracker (THP-1)
THP-1 recruitment (% of control)
300
200
100
0
#
*
*
L-NAME (mM)
-
-
0.1
LPS
Ad-GFP
Ad-HIF-2α

adipocytes that overexpressed HIF-2α in the presence of NOS inhibitor (Fig. 11C). In conditioned medium, it is likely that NO would be rarely present because of the short half-life of NO (only a few seconds). Therefore, decrease of macrophage migration upon NOS inhibitor would not be resulted from decrease of NO contents, present in conditioned medium. Rather, it appears that secretion of certain cytokine mediators from HIF-2α-overexpressing adipocytes might be interfered by NOS inhibitor. Therefore, macrophage migration by adipocyte HIF-2 seems to be mediated through certain mediators, tightly regulated by NO signaling.

Next, we examined whether adipocyte HIF-2α could influence on polarity of recruited macrophage or not. The mRNA levels of M2 markers such as IL-10, MGL1, MGL2, and MRC1 were increased in macrophages, which were cultured with conditioned medium from adipocytes (Fig. 12A), whereas M1 markers such as TNFα, IL-1β, and IL-6 was not changed or slightly increased (Fig. 12B). This result was probably the effects of Th2 cytokines released from adipocytes and involved in M2 polarization of macrophage (Kang et al., 2008). As shown in Fig. 12A, conditioned medium from HIF-2α-overexpression adipocytes neither accelerated M2 polarization of macrophages nor inhibited M2 polarization. Unlike LPS which induced the expression of pro-inflammatory cytokines in macrophages, conditioned medium from HIF-2α-overexpression adipocytes did not stimulate the expression levels of pro-inflammatory cytokines (Fig. 12B). These data imply that adipocyte HIF-2α should participate in macrophages recruitment with a little impact on the polarization of macrophages related to adipose tissue inflammation.

Figure 11. Conditioned medium from HIF-2α-overexpressing adipocytes stimulates macrophage migration. A: Illustration of migration assay with peritoneal macrophages. Peritoneal macrophages seeded on 8.0 μm pore trans-well were incubated with conditioned medium from Ad-GFP- or Ad-HIF-2α-infected adipocytes as described in Materials and Methods. LPS was used as positive-control B: After 1 hr, macrophages were harvested and total lysates were blotted with antibodies for detection of phosphorylation of p38 and ERK. C: For migration of macrophages, DAPI signals were observed using a Zeiss LSM510NLO confocal microscope. Quantitative measurements of migrated macrophages were obtained by Zeiss LSM Image Browser software. Data represent mean ± SD. $^{*}P < 0.05$ vs. Ad-GFP control with no treatment; $^{\#}P < 0.05$ vs. Ad-HIF-2α control with no treatment by Student's *t*-test.

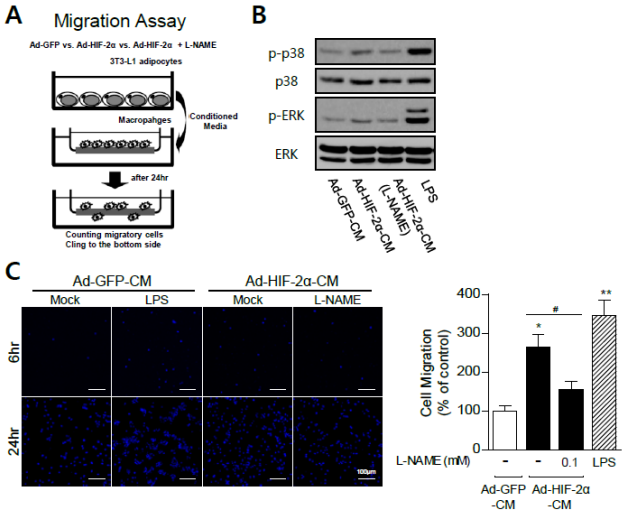
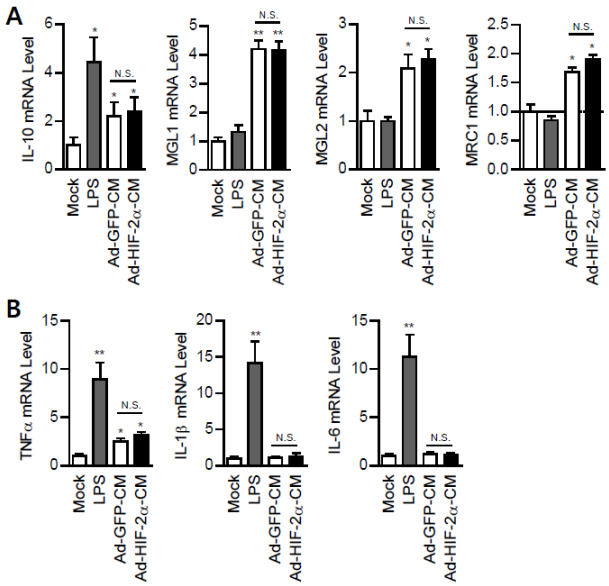

A
Migration Assay
Ad-GFP vs. Ad-HIF-2α vs. Ad-HIF-2α + L-NAME
3T3-L1 adipocytes
Macropahges
Conditioned Media
after 24hr
Counting migratory cells
Cling to the bottom side
B
p-p38
p38
p-ERK
ERK
Ad-GFP-CM
Ad-HIF-2α-CM
Ad-HIF-2α-CM (L-NAME)
LPS
C
Ad-GFP-CM
Ad-HIF-2α-CM
Mock
LPS
Mock
L-NAME
6hr
24hr
100μm
Cell Migration (% of control)
400
300
200
100
0
*
#
**
L-NAME (mM)
-
-
0.1
LPS
Ad-GFP -CM
Ad-HIF-2α -CM

Figure 12. Conditioned medium from HIF-2α-ocerexpressed adipocytes does not affect macrophage polarity. A-B: Conditioned medium from Ad-GFP- or Ad-HIF-2α-infected 3T3-L1 adipocytes treated in peritoneal macrophage for 24 hr. As negative control, peritoneal macrophages were cultured in fresh media for 24hr; Mock. And peritoneal macrophages cultured in fresh media were treated with 10 ng/ml LPS for pro-inflammatory stimulation for 24 hr. To detect macrophage polarity, total RNA was isolated and analyzed by qRT-PCR for M2 marker (IL10, MGL1, MGL2, and MRC1) (A) and pro-inflammatory cytokines (TNFα, IL1β, and IL6) (B). All mRNA expression levels were normalized by the level of cyclophilin mRNA. Data represent mean ± SD. **P* < 0.05, and ***P* < 0.01 vs. Mock control; by Student's *t*-test.


A
IL-10 mRNA Level
MGL1 mRNA Level
MGL2 mRNA Level
MRC1 mRNA Level
N.S.
Mock
LPS
Ad-GFP-CM
Ad-HIF-2α-CM
B
TNFα mRNA Level
IL-1β mRNA Level
IL-6 mRNA Level

HIF-2α-overexpressed adipocytes do not alter lipid metabolism, cellular lipid contents, oxidative stress, or insulin signaling

Recently, it has been suggested that adipose tissue hypoxia contributes to change in lipid metabolism, cellular oxidative stress, and insulin resistance in adipocytes (Regazzetti et al., 2009; Yin et al., 2009). To test whether HIF-2α is related to above functions in adipocytes, I have investigated the expression levels of genes related with lipid synthesis (sterol regulatory element-binding transcription factor 1; SREBP1c, acetyl-CoA carboxylase 1; ACC1, fatty acid synthase; FAS, and stearoyl-CoA desaturase-1; SCD1), lipid oxidation (peroxisome proliferator-activated receptor coactivaotr 1α; PGC1α, aconitase 1; ACO1, medium-chain acyl-CoA dehydrogenase; mCAD, and carnitine palmitoyltransferase 1α; CPT1α), lipid uptake (CD36, low density lipoprotein receptor; LDLR, and very low density lipoprotein receptor; VLDLR), and lipolysis (hormone-sensitive lipase; HSL, adipose triglyceride lipase; ATGL, and lipoprotein lipase; LPL) with or without HIF-2α overexpression in adipocytes. Expression profiles from qPCR analysis revealed that these genes were not significantly affected by HIF-2α overexpression (Fig. 13A~D). Consistently, cellular triglyceride contents also were not altered in Ad-HIF-2α-infected adipocytes compared to Ad-GFP-infected adipocytes (Fig. 13E). These data suggest that adipocyte HIF-2α might not be involved in is lipid metabolism. Further, HIF-2α overexpression did not induce cellular reactive oxidative species (ROS) in adipocytes although HIF-2α overexpression induced a few inflammatory genes and NO production (Fig. 14A). Similarly, the expression

levels of pro-oxidant genes (NADPH oxidase2; NOX2, NADPH oxidase4; NOX4, p22phox, p47phox, and p67phox) and anti-oxidant genes (glutathione peroxidase; GPx and Superoxide dismutase 2; SOD2) were not altered by HIF-2α overexpression (Fig. 14B and C). Additionally, there were no changes in insulin-stimulated phosphorylation of Akt and GSK3β, as downstream signaling molecules of insulin, or insulin-stimulated glucose uptake activity in HIF-2α-overexpressed adipocytes (Fig. 14D~F). Therefore, the induction of inflammatory mediators such as IL-6, SAA, and iNOS by HIF-2α in adipocytes may not be sufficient to induce adipocyte dysfunction, implying that adipocyte HIF-2α-mediated inflammatory response would be limited without additional metabolic stresses.

Adipocyte-macrophage interaction induces the expression of pro-angiogenic factors in macrophages

Recent studies about macrophage infiltration into adipose tissue have focused mostly on pro-inflammatory response in obesity (Lumeng et al., 2007a; Weisberg et al., 2003; Xu et al., 2003), by contrast, the angiogenic function of adipose tissue macrophage is not yet well-established. To examine whether macrophages recruited by adipocyte HIF-2α could participated in angiogenesis, I adopted indirect co-culture experiments to mimic the microenvironments of adipose tissue. As illustrated in figure 15A, 3T3-L1 adipocytes were indirectly co-cultured with macrophages, and pro-angiogenic genes were analyzed from either adipocytes or macrophages compared to independently cultured cells. Although the expression

Figure 13. HIF-2α overexpression does not change gene expression related to lipid metabolism and cellular lipid contents in adipocytes. A-D: 3T3-L1 adipocytes were infected with Ad-HIF-2α or Ad-GFP. After 48 hr, total RNA was isolated and analyzed by qRT-PCR for lipogenic genes (SREBP1c, ACC1, FAS, and SCD1) (A), lipid oxidation genes (PGC1α, ACO1, mCAD, and CPT1α) (B), lipid uptake genes (CD36, LDLR, and VLDLR), and lipolysis genes (HSL, ATGL, and LDL) (D). All mRNA expression levels were normalized by the level of cyclophilin mRNA. Data represent mean ± SD. $*P < 0.05$ vs. Ad-GFP control by Student's *t*-test. Total cell lysates were collected from Ad-GFP- or Ad-HIF-2α-infected adipocytes and thus cellular triglyceride level was measured (E). The data were normalized by using protein concentrations. Data represent mean ± SD. $*P < 0.05$ vs. Ad-GFP control by Student's *t*-test.

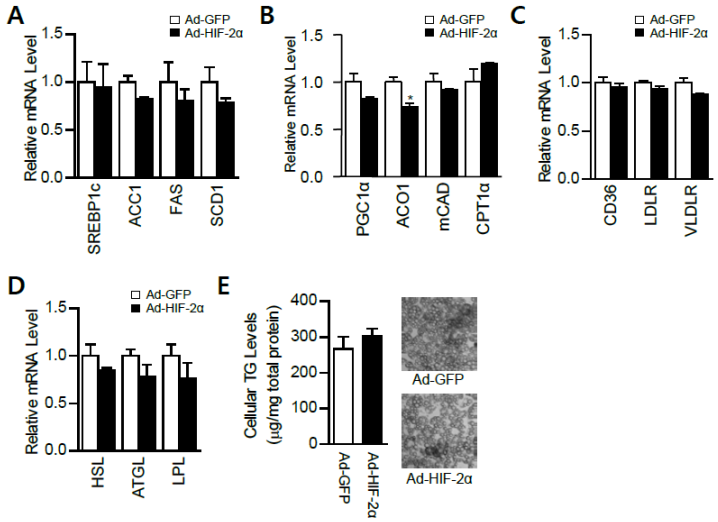

A
Ad-GFP
Ad-HIF-2α
Relative mRNA Level
1.5
1.0
0.5
0.0
SREBP1c
ACC1
FAS
SCD1
B
Ad-GFP
Ad-HIF-2α
Relative mRNA Level
1.5
1.0
0.5
0.0
*
PGC1α
ACO1
mCAD
CPT1α
C
Ad-GFP
Ad-HIF-2α
Relative mRNA Level
1.5
1.0
0.5
0.0
CD36
LDLR
VLDLR
D
Ad-GFP
Ad-HIF-2α
Relative mRNA Level
1.5
1.0
0.5
0.0
HSL
ATGL
LPL
E
Cellular TG Levels
(μg/mg total protein)
400
300
200
100
0
Ad-GFP
Ad-HIF-2α
Ad-GFP
Ad-HIF-2α

Figure 14. HIF-2α overexpression does not alter cellular ROS level and insulin signaling in adipocytes. A-D: 3T3-L1 adipocytes were infected with Ad-HIF-2α or Ad-GFP. Cellular ROS in these adipocytes was detected using DCF-DA, which generates a fluorescent signal that is visualized by a conforcal microscope (A) Total RNA was isolated and analyzed by qRT-PCR for pro-oxidative genes (NOX2, NOX4, p22phox, p47phox, and p67phox) (B), anti-oxidative genes (GPx and SOD2) (C) and insulin signaling-related genes (IR, IRS2, GLUT4) (D). All mRNA expression levels were normalized by the level of cyclophilin mRNA. Data represent mean ± SD. **P* < 0.05 vs. Ad-GFP control by Student's *t*-test. E-F: For measure insulin signaling and glucose uptake, adipocytes infected with Ad-HIF-2α or Ad-GFP with or without L-NAME were incubated in serum-free low glucose DMEM for 8hrs. After 30 min of stimulation with insulin (50 nM), adipocytes were harvested and total lysates were blotted with antibodies for detection of HIF-2α, iNOS and insulin signaling cascade (pAkt, total Akt, pGSK3β, pGSK3β). β-actin was used as the loading control (D). Glucose uptake assay was performed as decribed in Materials and Methods. Relative fold of glucose uptake was shown in graph. Rosiglitazone (rosi) was used for positive contol (F). Data represent mean ± SD. ***P* < 0.01 vs. no treat of insulin control by Student's *t*-test.

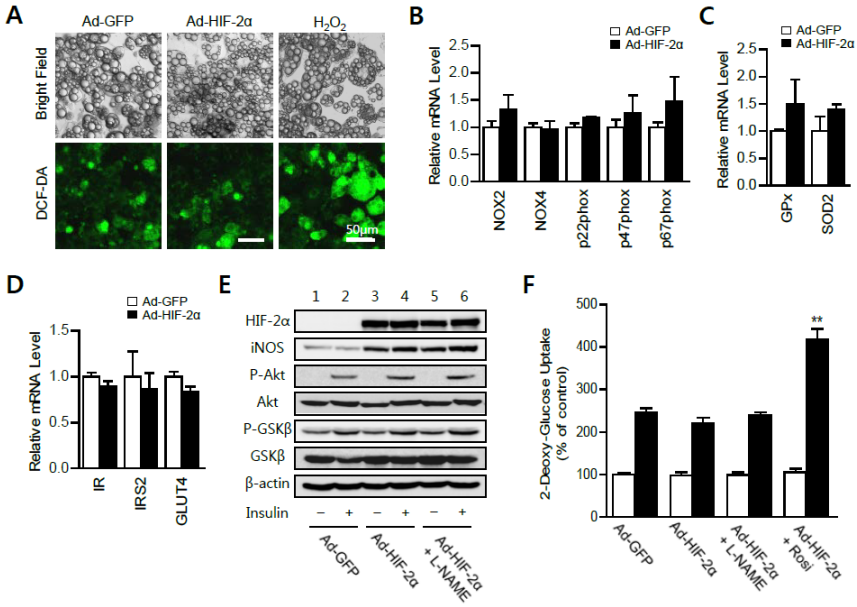

A
Ad-GFP
Ad-HIF-2α
H2O2
Bright Field
DCF-DA
50μm
B
Ad-GFP
Ad-HIF-2α
Relative mRNA Level
NOX2
NOX4
p22phox
p47phox
p67phox
C
Ad-GFP
Ad-HIF-2α
Relative mRNA Level
GPx
SOD2
D
Ad-GFP
Ad-HIF-2α
Relative mRNA Level
IR
IRS2
GLUT4
E
1 2 3 4 5 6
HIF-2α
iNOS
P-Akt
Akt
P-GSKβ
GSKβ
β-actin
Insulin
Ad-GFP
Ad-HIF-2α
Ad-HIF-2α + L-NAME
F
2-Deoxy-Glucose Uptake (% of control)
**
Ad-GFP
Ad-HIF-2α
Ad-HIF-2α + L-NAME
Ad-HIF-2α + Rosi

Figure 15. The expression levels of IGF1 and PDGFβ are increased in macrophages by co-culture with adipocytes. A: Adipocytes and macrophages were cultured each or together using indirect co-culture system with 0.4 μm pore trans-well as illustration (A) After 24 hr, adipocytes and macrophage was harvested and used to extract total RNA. B: The relative mRNA level of pro-angiogenic factors (VEGFα, FGF2, HGF, IGF1, ANGPTL4, and PDGFβ) were analyzed by qRT-PCR in co-cultured adipocytes (B) and co-cultured macrophages (C) compared to control. The mRNA expression levels were normalized by the level of cyclophilin mRNA. Data represent mean ± SD. **P* < 0.05, and ***P* < 0.05 vs. single cultured control by Student's *t*-test.

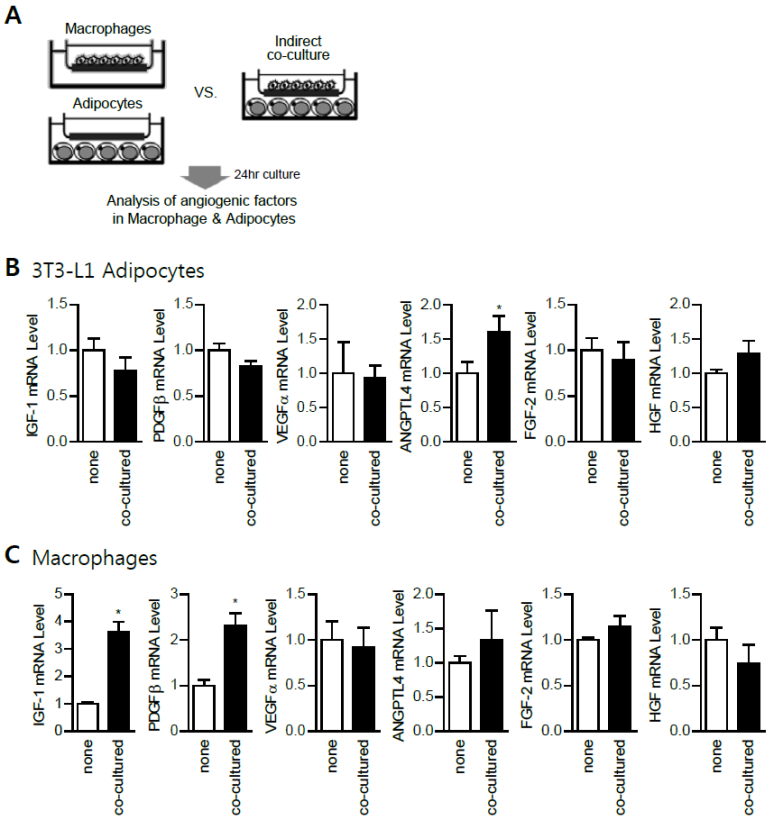
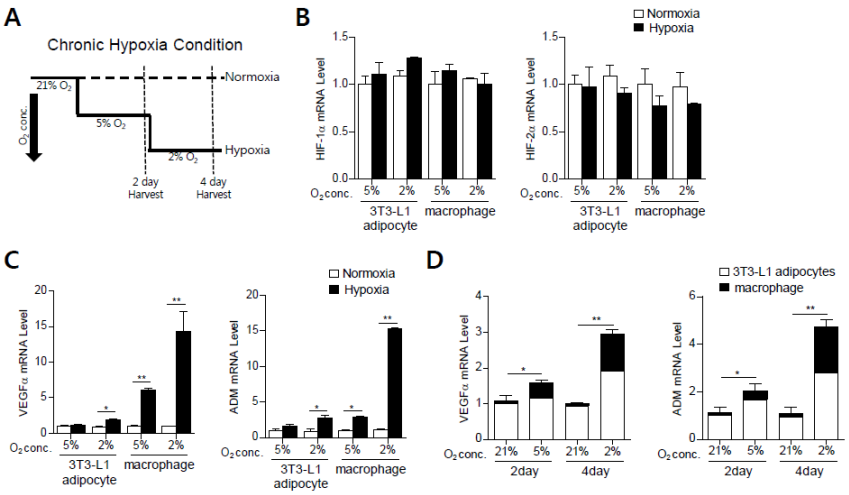

A
Macrophages
Adipocytes
VS.
Indirect co-culture
24hr culture
Analysis of angiogenic factors in Macrophage & Adipocytes
B 3T3-L1 Adipocytes
IGF-1 mRNA Level
PDGFβ mRNA Level
VEGFα mRNA Level
ANGPTL4 mRNA Level
FGF-2 mRNA Level
HGF mRNA Level
none
co-cultured
C Macrophages
IGF-1 mRNA Level
PDGFβ mRNA Level
VEGFα mRNA Level
ANGPTL4 mRNA Level
FGF-2 mRNA Level
HGF mRNA Level
none
co-cultured

Figure 16. Induction of VEGFα and ADM by hypoxia is more sensitive in macrophage than in adipocytes. A-D: Each of adipocytes and macrophages were cultured in normoxic condition and chronic hypoxic condition, where oxygen concentration was dropped to 5%, and then to 1% with 2 days interval. On the fourth day, each of adipocytes and macrophage were harvested and used to extract total RNA (A). The relative mRNA level of HIF family (HIF-1α and HIF-2α) (B), and pro-angiogenic factors (VEGFα and ADM) (C) were analyzed by qRT-PCR in each of adipocytes and macrophages. The mRNA level of each genes were normalized by the level of cyclophilin mRNA and presented fold of activity compared to the mRNA level of each genes in each simultaneous normoxic control cells. Data represent mean ± SD. *$P < 0.05$, and **$P < 0.05$ vs. normoxia control by Student's *t*-test. Sum of the mRNA level in adipocytes and macrophages of VEGFα or ADM was respectively presented in graph (D). The mRNA expression levels were normalized by the level of cyclophilin mRNA. Data represent mean ± SD. *$P < 0.05$, and **$P < 0.05$ vs. normoxia control by Student's *t*-test.


A
Chronic Hypoxia Condition
21% O2
5% O2
2% O2
Normoxia
Hypoxia
O2 conc.
2 day Harvest
4 day Harvest
B
Normoxia
Hypoxia
HIF-1α mRNA Level
HIF-2α mRNA Level
O2 conc.
5% 2% 5% 2%
3T3-L1 adipocyte
macrophage
C
VEGFα mRNA Level
ADM mRNA Level
D
3T3-L1 adipocytes
macrophage
21% 5% 21% 2%
2day
4day

levels of pro-angiogenic genes in adipocytes were not significantly changed, the expression levels of IGF-1 and PDGFβ in macrophages were remarkably increased when macrophages were co-cultured with adipocytes (Fig. 15B and C). In addition, to examine the pro-angiogenic phenotype of macrophage depending on hypoxic condition, each adipocytes and macrophages were sequentially and gradually exposed to chronic hypoxic conditions; 2 days at 5% O_2 and then 2 days at 2% O_2 (Fig. 16A). Interestingly, the mRNA levels of pro-angiogenic factors, VEGFα and ADM, were highly and sensitively elevated in macrophages more than in adipocytes in both mild hypoxic condition (5%) and severe hypoxic condition (2%) without transcriptional change of HIF family (Fig. 16B and C). Considering the absolute values of VEGFα and ADM transcripts, the mRNA levels of these genes in macrophages were very low compared to those in adipocytes. However, mRNA levels of those genes were drastically induced in macrophages by chronic hypoxia so as to be similar to their amounts in adipocytes (Fig. 16D). These data suggest that the inducible regulation of VEGFα and ADM in macrophages might contribute to fine-tuning of angiogenesis in adipose tissue depending on local hypoxic microenvironments. Taken together, these data propose the idea that macrophages recruited into adipose tissue may have pro-angiogenic function in early stage of obesity.

DISCUSSION

In the progress of obesity, sufficient development of vascular network in adipose tissue contributes to the prevention of hypoxia, which is involved in adipose tissue inflammation, fibrosis, and insulin resistance (Sun et al., 2011; Sung et al., 2013). Of interest, it has been reported that the vascular network of adipose tissue is a potential source of adipocyte progenitors and a platform for adipogenesis, which assists in the supply of small neo-adipocytes (Cho et al., 2007; Rutkowski et al., 2009; Tang et al., 2008). Thus, the regulation of pro-angiogenic factors in adipose tissue upon metabolic status is a key factor for healthy adipose tissue expansion. HIF-2α is one of key transcription factors to induce pro-angiogenic factors, and expression of HIF-2α is increased during adipogenesis as well (Keith et al., 2012; Ratcliffe, 2007). However, in adipocytes, the role of HIF-2α as a regulator of pro-angiogenic factors has not been clearly understood. In this study, elevated adipocyte HIF-2α in response to adipogenic, hypertrophic, and hypoxic stimulation could promote the expression of pro-angiogenic factors including VEGFα and recruitment of macrophage to support angiogenesis.

During excess caloric intake, adipose tissue exhibits remarkable plasticity in that it is able to expand in company with enlargement of adipocytes (Lee et al., 2011b; Sun et al., 2011). In a short-term HFD mice model, hypertrophic adipocytes were observed only after 1 week (Fig. 5), as shown in previous report (Lee et al., 2011b). Concurrently, both the numbers of adipose tissue macrophages (ATMs) and the mRNA levels of ATM markers were elevated in adipose tissue by 1 week HFD

(Fig. 7 and 8). However, it has not been thoroughly studied how quickly adipose tissue vasculature is able to change in response to the expansion of adipose tissue upon HFD. As shown in Fig. 7, the density of adipose vasculature was not altered in spite of adipose tissue expansion and adipocyte enlargement at 1 week of HFD. The maintenance of blood vessel density in adipose tissue of 1 week HFD-fed mice allowed hypertrophic adipocytes becoming into contact with relatively more blood vessels compared to small adipocytes (Fig. 7). Consistently, the expression levels of endothelial cell markers were increased in adipose tissue from 1 week HFD-fed mice, which was expected to be the consequence of angiogenesis considering the significant enrichment of genes linked to VEGFα signaling (Fig. 8). Interestingly, in the early period of HFD, HIF-2α protein as well as its mRNA was increased in hypertrophic adipocytes (Fig. 6), which proposes the possibility that adipocyte HIF-2α may be a transcription factor mediating angiogenesis in adipose tissue of short-term HFD-fed mice. Recently, the post-translational regulation of HIF-2α by hypoxia has been reported, whereas relatively little is known about the transcriptional regulation of HIF-2α gene (Keith et al., 2012; Ratcliffe, 2007). In this work, *in vitro* experiments showed that the induction of adipocyte HIF-2α mRNA would be associated with adipogenic and hypertrophic stimulation (Fig. 4). Consequently, transcriptionally up-regulated of HIF-2α seems to actively participate in adipose tissue angiogenesis at the early stage of obesity with intermittent hypoxia.

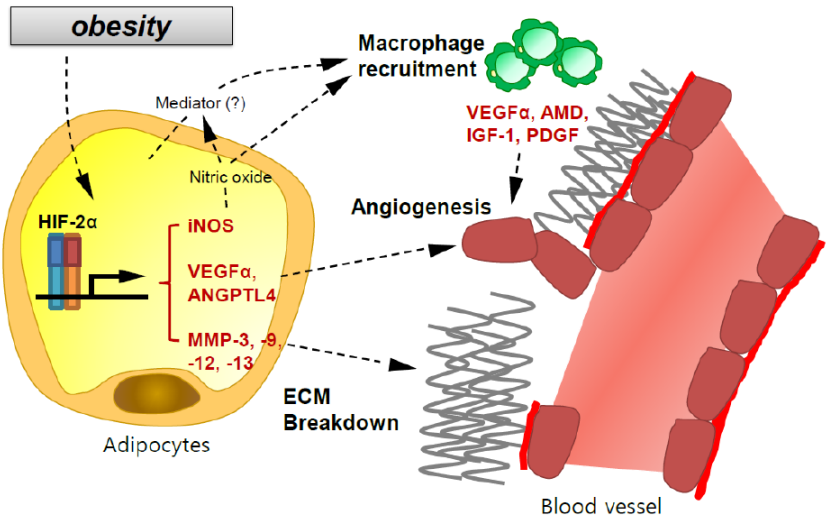
One of the interesting observations was that HIF-2α-overexpressed adipocytes exhibited significantly increased expression levels of MMP-3, MMP-9,

and MMP-13 (Fig. 9), which were reported to be up-regulated in expanding adipose tissue of obese subjects (Christiaens and Lijnen, 2006; Unal et al., 2010). Accumulating evidences show that the breakdown and/or degradation of basement membrane proteins is an essential process in the initiation of angiogenesis, which is mediated by matrix metalloproteinases (MMPs) (Pepper, 2001; Yancopoulos et al., 2000). Indeed, several studies have suggested that MMPs including MMP-9 potentially affect microvessel maturation and adipogenesis in adipose tissue via modulating extracellular matrix (ECM) (Lijnen et al., 2002; Van Hul and Lijnen, 2011). Based on these results, it is possible to suggest that adipocyte HIF-2α should be not only a transcriptional regulator for expression of pro-angiogenic factors but also a modulator of adipose tissue structure for angiogenesis. Furthermore, MMP-9, having gelatinase activity, is able to release the matrix-bound VEGFα isoform (Bergers et al., 2000). These findings propose the idea that the target genes of HIF-2α synergistically would promote adipose tissue angiogenesis. Additionally, it appears that NO induced by adipocyte HIF-2α might be also involved in the modulation of adipose tissue angiogenesis. It has been demonstrated that the physiological level of NO has various functional roles as a signaling molecule with regard to the vascular permeability, oxygenation, blood flow, growth, and density of blood capillaries (Schwentker et al., 2002; Singh and Agarwal, 2007). In this study, HIF-2α overexpression in adipocytes mildly induced NO production about two-fold via the up-regulation of iNOS (Fig. 9), which is quite different from high concentration of NO stimulated in macrophages during LPS-induced acute

inflammation or long-term HFD-induced chronic inflammatory responses. Indeed, iNOS induction by HIF-2α in adipocytes did not cause significant cellular damages such as oxidative stress accumulation or insulin resistance (Fig. 14). Thus, it is feasible to propose that adipocytes HIF-2α would participate in overall adipose tissue remodeling upon HFD through induction of its target genes.

In solid tumors or wound repairing processes, recruited macrophages have been reported to play a key role in angiogenesis (Deonarine et al., 2007; Jeon et al., 2007; Sica et al., 2006; Tjiu et al., 2009; van der Plas et al., 2009). Similarly, recent studies have suggested that ATMs are involved in the process of adipose tissue development through the extension of vascular structure (Bourlier et al., 2008; Cho et al., 2007). With regard to the role of macrophage recruited during angiogenesis, it is of interest that HIF-2α-overexpressing adipocytes exhibited the promotion of macrophage recruitment and migration, which seemed to be associated with the direct and indirect effects of NO produced by HIF-2α-induced iNOS (Fig. 10 and 11). Although increased macrophages in adipose tissue are responsible for metabolic disorders as a source of pro-inflammatory cytokines in obesity, several lines of evidence suggest that recruited ATMs would participate in simulating angiogenesis in adipose tissue as well. First, IGF-1 and PDGFβ, key angiogenic factors, were induced in macrophages co-cultured with adipocytes (Fig. 15). Together with VEGFα, IGF-1 is one of the most important angiogenic and survival factors for endothelial cells (Seccareccia and Brodt, 2012). PDGFβ, a homologue of VEGFα, also helps to maintain capillary wall stability (Papetti and Herman, 2002).

Figure 17. Proposed model for role of adipocyte HIF-2α in obesity-induced adipose tissue angiogenesis. In obesity, activation of adipocyte HIF-2α is suggested to promote angiogenesis, extracellular matrix (ECM) breakdown, and macrophage recruitment via transcriptional activation of target genes, which regulate adipose tissue remodeling in response to metabolic condition.


obesity
Macrophage recruitment
Mediator (?)
Nitric oxide
VEGFα, AMD, IGF-1, PDGF
Angiogenesis
HIF-2α
iNOS
VEGFα, ANGPTL4
MMP-3, -9, -12, -13
ECM Breakdown
Adipocytes
Blood vessel

Second, macrophages carry out inducible regulation of VEGFα and ADM genes upon hypoxic condition (Fig. 16). In normoxic condition, the mRNA levels of VEGFα and ADM in macrophages were lower than that in adipocytes. However, the induction of VEGFα and ADM mRNA in macrophages was susceptible to both mild and severe hypoxia and their induction folds were also greater than in adipocytes. These results imply that recruited macrophages into adipose tissue during obesity would efficiently support angiogenesis only in the small area where local hypoxia occurred. Third, macrophages exhibited anti-inflammatory M2 phenotypes by incubating with conditioned medium from adipocytes (Fig. 12). Recent studies suggest that M2 macrophages are mainly related to repair or remodeling of tissues (Fujisaka et al., 2009; Odegaard and Chawla, 2011; Shaul et al., 2010). These data imply that adipocytes and macrophages could regulate the expression of multiple pro-angiogenic factors through functional interactions to accommodate vessel growth and homeostasis.

Although future studies will reveal how adipocyte HIF-2α could contribute to adipose tissue angiogenesis *in vivo*, the data presented here suggest that adipocyte HIF-2α might systemically modulate adipose tissue remodeling for angiogenesis via cross-talk between adipocytes and macrophages (Fig. 17). In addition, rapid and dynamic adipose tissue remodeling in mice was induced by this short-term HFD, accompanied adipocyte HIF-2α accumulation, which proposed the possibility that adipocyte HIF-2α might be concerned in adipose tissue remodeling in very early stage of obesity.

CHAPTER TWO:

Macrophage hypoxia-inducible factor 2α ameliorates adipose tissue inflammation and insulin resistance in obesity

Abstract

In obesity, adipose tissue macrophages (ATMs) play a key role to mediate pro-inflammatory responses in adipose tissue, which is associated with obesity-related metabolic complications. Recently, adipose tissue hypoxia has been implicated in the regulation of ATMs in obesity. However, the role of hypoxia-inducible factor 2α (HIF-2α) which is one of the major transcription factors induced by hypoxia has not been thoroughly elucidated in ATMs. In this study, I demonstrate that elevation of macrophage HIF-2α would attenuate adipose tissue inflammation and improve insulin resistance in obesity. In macrophages, overexpression of HIF-2α decreased nitric oxide production and suppresses expression of pro-inflammatory cytokines through induction of arginase 1 (ARG1). HIF-2α-overexpressing macrophages alleviated pro-inflammatory responses and improved insulin resistance in adipocytes. In contrast, knock-down of macrophage HIF-2α augmented palmitate-induced pro-inflammatory gene expression in adipocytes. Furthermore, compared to wild-type mice, HIF-2α heterozygous-null mice aggravated insulin resistance and adipose tissue inflammation with more M1-like ATMs upon high-fat diet (HFD). Moreover, glucose intolerance in HFD-fed HIF-2α heterozygote mice was relieved by macrophage deletion with clodronate treatment, implying that HIF-2α in ATMs would contribute to the resolution of adipose tissue inflammation and insulin resistance in obesity. Taken together, these data suggest that macrophage HIF-2α would suppress pro-inflammatory responses of ATMs against obesity-induced insulin resistance in adipose tissue.

Introduction

Accumulating evidence has proposed that obesity is characterized by chronic and low-grade inflammation accompanied by macrophage accumulation in adipose tissue, which eventually leads to metabolic diseases including insulin resistance and type 2 diabetes (Donath and Shoelson, 2011; Olefsky and Glass, 2010; Wellen and Hotamisligil, 2003). Increased adipose tissue macrophages (ATMs) play crucial roles in the altered production of pro-inflammatory cytokines in fat tissue of obesity (Lumeng et al., 2007b; Weisberg et al., 2003; Xu et al., 2003). In healthy lean mice, ATMs are mostly comprised of alternatively activated (M2) type macrophages, which express high levels of anti-inflammatory cytokines such as IL-10 and specific enzymes such as arginase 1 (ARG1) in company with low levels of pro-inflammatory signals (Fujisaka et al., 2009; Lumeng et al., 2007a). Thus, M2 ATMs contribute to metabolic homoeostasis by keeping down adipose tissue inflammation (Fujisaka et al., 2009; Lumeng et al., 2007a; Sun et al., 2011). In the progress of obesity, some of ATMs are polarized from M2 type to classically activated (M1) type (Fujisaka et al., 2009; Lumeng et al., 2007a; Sun et al., 2011). In obese adipose tissue, M1 ATMs stimulate nitric oxide (NO) production with increase of iNOS and secretion of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), and IL-1β. Thereby, the balance shifting between M1 and M2 ATMs appears to be important for inflammatory responses in adipose tissue of obesity.

In adipocytes, pro-inflammatory cytokines secreted from M1 ATMs

repress insulin action by activation of IκB kinase-β (IKKβ) and JUN N-terminal kinase (JNK). These stress-activated kinases induce serine phosphorylation of insulin receptor substate 1 (IRS1) and IRS2, the key downstream mediators of insulin receptor (Chawla et al., 2011; Ferrante, 2007; Olefsky and Glass, 2010; Tilg and Moschen, 2008). In addition, IKKβ and JNK augment pro-inflammatory transcription factors such as nuclear factor-κB (NFκB) to further stimulate expression of pro-inflammatory genes, which leads to insulin resistance (Odegaard and Chawla, 2011; Wellen and Hotamisligil, 2005). On the other hand, it has been recently reported that M2 ATMs are also elevated in obesity to repair and/or remodel damaged adipose tissues primarily provoked by increased pro-inflammatory M1 ATMs (Fujisaka et al., 2009; Odegaard and Chawla, 2011; Shaul et al., 2010). Thus, alternative activation of M2 ATMs by Th2 cytokines such as IL-4 and IL-13 is known to be a resolution power to relieve metabolic dysregulation and restores insulin sensitivity (Kang et al., 2008; Odegaard et al., 2008; Ricardo-Gonzalez et al., 2010).

In obese adipose tissue, M1 polarization of ATMs attributes to various changes of local microenvironments (Sun et al., 2011). Among them, adipose tissue hypoxia is one of tissue specific phenomenon occurred with the rapid expansion of adipose tissue in obesity. Patho-physiological role of ATMs is regulated by certain transcription factors induced by hypoxia in combination with metabolic stresses (Hosogai et al., 2007; Sun et al., 2011; Ye et al., 2007). In adipose tissues, hypoxia-inducible transcription factor (HIF) 1α and 2α are key transcription factors to

mediate hypoxic responses such as angiogenesis, glycolysis, adhesion, and infiltration (Fang et al., 2009). Until now, most studies for adipose tissue HIFs have been focused on HIF-1α. For instance, activation of HIF-1α has been implicated in adipose tissue inflammation, fibrosis, and adipocyte dysfunction (Fujisaka et al., 2013; Halberg et al., 2009; Jiang et al., 2011; Lee et al., 2011a). In addition, it has been suggested that HIF-1α is involved in pro-inflammatory feature of ATMs in obesity (Fujisaka et al., 2013). However, although HIF-1α has been extensively investigated in adipose tissue inflammation, the role of HIF-2α, also known as endothelial PAS domain protein 1 (EPAS1), is largely unknown in obesity.

HIF-2α shares many features with HIF-1α in terms of structure, reactivity to hypoxia, DNA binding motif (so called hypoxia-response element; HRE) and target genes such as glucose transporter 1 (GLUT1), vascular endothelial growth factor alpha (VEGFα) and adrenomedullin (ADM) (Patel and Simon, 2008). However, despite of extensive homology to HIF-1α, HIF-2α has its own physiological roles via induction of unique target genes such as erythropoietin (EPO), octamer-binding transcription factor 4 (OCT4), and delta like ligand 4 (DLL4) (Keith et al., 2012). Furthermore, the works for HIF-1α and HIF-2α in solid tumors have revealed their opposing roles in cell growth, energy metabolism, NO homeostasis, and others. (An et al., 1998; Bertout et al., 2009; Gordan et al., 2007; Keith et al., 2012; Koshiji et al., 2004). For example, HIF-2α promotes cell proliferation by enhancing oncogene c-Myc activity in hypoxic tumor growth, whereas HIF-1α induces cell cycle arrest by inhibition of c-Myc activity (Gordan et

al., 2007; Koshiji et al., 2004). In addition, HIF-2α suppresses the p53 pathway, a crucial tumor suppressor, but HIF-1α promotes p53 (An et al., 1998; Bertout et al., 2009). These findings led us to test the idea whether HIF-2α may have distinctive roles to HIF-1α in adipose tissue of obesity although both HIF-1α and HIF-2α might be involved in angiogenesis in response to adipose tissue hypoxia. In particular, it is largely unknown the functional roles of HIF-2α in the regulation of inflammatory responses of ATMs to modulate adipose tissue inflammation.

In this study, I have demonstrated that macrophage HIF-2α attenuates pro-inflammatory property via induction of ARG1, which would prevent pro-inflammatory responses and insulin resistance in adipocytes. Consistently, haploid deficiency of HIF-2α in mice exacerbated adipose tissue inflammation and insulin resistance in high-fat diet (HFD)-fed obesity. In addition, depletion of ATMs by clodronate injection restored insulin sensitivity in HFD-fed HIF-2α heterozygote mice, implying that the increase of pro-inflammatory ATMs in severe obesity would be a major criminal for insulin resistance in HIF-2α heterozygote mice. Collectively, these data suggest that macrophage HIF-2α would alleviate insulin resistance in adipose tissue of obesity through suppression of pro-inflammatory responses of ATMs induced by metabolic stresses.

Materials and Methods

Animals and treatment. *db/db* and *db/+* mice were purchased from Central Lab Animal Inc, South Korea, and sacrificed at 12 weeks of age for detection of HIF-2α in adipose tissue. HIF-2$\alpha^{+/-}$ mice were generously provided by Jang-Soo Chun. They were maintained under pathogen-free conditions, and were housed in solid-bottom cages with wood shavings for bedding in a room maintained at 25°C with a 12:12 hr light: dark cycle (lights on at 07:00). Heterozygous mice were bred to generate HIF-2$\alpha^{+/-}$ mice and wild-type (WT) littermates. HIF-2$\alpha^{+/-}$ and WT mice were maintained on normal diet (ND) until 10 weeks of age and then were fed high-fat diet (HFD; 60% of calories derived from fat; Research Diets Inc., New Brunswick, NJ) for 12~16 weeks. For the oral glucose tolerance test, mice were fasted for 6 hrs and administrated with glucose (3 g/kg of body weight; Sigma-Adrich, MO). Blood glucose levels were measured at indicated time point with a freestyle blood glucose meter (Therasense; Uppsala, Sweden). For the insulin tolerance test, mice were fasted for 3 hrs and injected with insulin (1 unit/kg of body weight; Lilly, IN) and then blood glucose levels were measured at indicated time point. For the macrophage depletion, clodronate liposome (FormuMax Scientific Inc., CA) was intraperitoneally injected two times with an interval of 3 days as recommended by the manufacturer. All mice were euthanized, dissected and tissue specimens were immediately stored at -80°C until analysis. All animal procedures were in accordance with the research guidelines of the Seoul National University Animal Experiment Ethics Committee.

Adipose tissue fractionation. Fractionation of adipose tissue was performed as previously described, with minor modifications (Park et al., 2006). Briefly, epididymal adipose tissues were digested in type I collagenase buffer and filtered through nylon mesh. After centrifugation, floating adipocytes fraction and pelleted stromal vascular cells (SVCs) fraction were washed several times and collected each fraction for RNA extraction. SVCs fraction was also used for flow cytometry.

Flow cytometry analysis. Flow cytometry analysis was performed as previously described (Huh et al., 2013). Red blood cells were removed from SVCs fraction using RBC lysis buffer. After incubation with blocking antibody, I stained SVCs with CD11b (BD Bioscience, CA), F4/80 (eBioscience), and CD11c (eBioscience) MAbs for macrophage analysis. SVCs were analyzed using the fluorescence-activated cell sorting (FACS) CantoII instrument (BD Bioscience). For sorting of CD11c-positive and negative macrophages ($CD11b^+$ and $F4/80^+$ respectively), FACS AriaII instrument (BD Bioscience) was used.

Whole-mount immunofluorescence. Whole-mount immunofluorescence was performed as previously described, with minor modifications (Huh et al., 2013). Epididymal adipose tissues were fixed with 1% paraformaldehyde and blocked with 5% goat serum in PBST for 1hr. For whole-mounted epididymal adipose tissue were incubated overnight at 4°C with primary antibody for HIF-2α (1:1000; Novus, CO), perilipin (1:1,000; Fitzgerald, MA), CD11b (1:1000; eBioScience), CD31 (1:1000; Millipore, MA). After being washed for 1 hr, the samples were incubated with

fluorescence-labeled secondary antibody for 4 hrs at room temperature and washed. Following staining with 4',6-diamidino-2-phenylindole (DAPI) (Vector Lab., CA), samples were observed using a Zeiss LSM510NLO confocal microscope. For detection of blood vessel, images gotten via z-section in 80 μm thickness adipose tissue stained with CD31 antibody were accumulated using Zeiss LSM Image Browser software.

Isolation of peritoneal macrophage. Mice were injected intraperitoneally with sterile fluid thioglycollate solution (2 ml per mouse). After 3 days, the peritoneal cells were harvested by washing the peritoneal cavity with PBS containing 5 mM EDTA. Primary peritoneal macrophages were cultured with Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT) with 10% fetal bovine serum (FBS; HyClone) to allow cell adherence. The non-adherent cells were removed by washing.

Adenovirus infection. HIF-2α adenovirus was generously provided by Jang-Soo Chun (Yang et al., 2010). As a negative control, GFP adenovirus (Neurogenex, South Korea) was used. Primary cultured peritoneal macrophages were incubated with serum-free DMEM and 100 MOI (multiplicity of infection) of adenovirus for 16 hrs. Then, the culture medium was replaced with fresh medium. Each experiment was performed at 48 hrs after viral infection.

Transfection with siRNA. small interfering RNA (siRNA) was delivered into 3T3-L1 adipocytes and peritoneal macrophage by electroporation. For this, peritoneal

macrophage was used as soon as primary cultured without attachment, and 3T3-L1 adipocytes were prepared to be detached by trypsine-EDTA after 4 day differentiation. The sequences of the siRNAs targeting HIF-2α were sense; 5'-CUCAGUUACAGCCACAUCGUCACUG-3', antisense; 5'-CAGUGACGAUGUGGCUGUAACUGAG-3' (Yang et al., 2010). As a negative control, scramble siRNA (siCTL) was used.

3T3-L1 adipocyte differentiation and co-culture experiment. 3T3-L1 preadipocytes were grown to confluence in DMEM supplemented with 10% bovine calf serum (HyClone). At 2 days after confluence, differentiation of the 3T3-L1 cells were stimulated with DMEM containing 10% FBS, methylisobutylxanthine (500 μmol/l), dexamethasone (1 μmol/l), and insulin (5 μg/ml) for 2 days. Then, culture medium was changed with DMEM containing 10% FBS and insulin (5 μg/ml). For indirect co-culture experiment, 3T3-L1 adipocytes were differentiated in the lower chamber of the well and then co-cultured with macrophages, which were modified gene expression of HIF-2α then prepared to grow on 0.4 μm pore trans-well. After 48 hrs of incubation, trans-well attached macrophages were removed and 3T3-L1 cells were used for RNA extraction, insulin-stimulated glucose uptake assay, and western blotting for insulin signaling detection after insulin treatment. For co-culturing macrophage with adipose tissue, epididymal adipose tissue was chopped about 1mm size and then 50 mg adipose tissues per wells co-cultured with 5 X 10^5 macrophages attached in the lower chamber of the well to be separated with by 0.4 μm pore trans-well.

Insulin-stimulated glucose uptake assay. Insulin stimulated glucose uptake in 3T3-L1 adipocytes was determined by measuring [^{14}C]-2-deoxy-glucose uptake as previously described, with minor modifications (Jeong et al., 2009). In brief, 3T3-L1 adipocytes co-cultured with macrophages were incubated in low-glucose DMEM containing 0.1% BSA for 8 hrs. Cells were stimulated with or without 50 nM insulin for 15 min. Glucose uptake was initiated by the addition of [^{14}C]-deoxy-D-glucose (PerkinElmer Life, MA) at a final concentration of 3 μmol/L for 15 min in HEPES buffer saline. The reaction was terminated by several cold-PBS washing. After cells were lysed with 0.1% SDS, ^{14}C radioactivity was measured using scintillation counter. Measured ^{14}C radioactivities were normalized by total protein concentration of whole lysates.

Quantitative RT-PCR. Total RNA was isolated from peritoneal macrophages, 3T3-L1 adipocytes and epididymal adipose tissues as described previously (Choe et al., 2007). cDNA was synthesized using the M-MuLV reverse transcriptase kit according to the manufacturers' protocol (Thermo Fisher Scientific, MA). The primers used for quantitative real-time PCR (Bioneer, South Korea) and their sequences are provided in supplementary Table I.

Western blot analysis. Western blot analysis was performed as described previously (Choe et al., 2007). Peritoneal macrophages, 3T3-L1 adipocytes and epididymal adipose tissues were lysed with NETN buffer. The proteins were separated on SDS-PAGE gels and transferred to PVDF membranes (Millipore).

Table 2. qRT-PCR primer sequences

Gene	*5′sequence*	*3′sequence*
ACC1	GAGTGACTGCCGAAACATCT	GCCTCTTCCTGACAAACGAG
ACO1	TGAAAACAGTTGTGCCTTGC	ACAGAGCCATGAGCGAGAGT
Adiponectin	GGCAGGAAAGGAGAGCCTGG	GCCTTGTCCTTCTTGAAGAG
AMD	TCAGAGCATCGCCACAGAAAT	TAGCTGCTGGATGCTTGTAG
ARG1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
ARG2	CTCGTTCAGTGGGCCTTG	GTCCGCATGAGCATCAAC
CD11c	CTGGATAGCCTTTCTTCTGC	GCACACTGTGTCCGAACTC
CD31	CTTGTCATCAACGGGAAGC	CAAAGTTGTCATGGATGACC
CHI3L3	AGAAGGGAGTTTCAACCTGGT	GTCTTGCTCATGTGTGTAAGTGA
Cyclophilin	CAGACGCCACTGTCGCTTT	TGTCTTTGGAACTTTGTCTG
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
FAS	CGGTAGCTCTGGGTGTA	TGCTCCCAGCTGCAGGC
GLUT1	ACTGGGCAAGTCCTTTGAGA	GTCTAAGCCAAACACCTGGGC
HIF-1α	CAAGATCTCGGCGAAGCAA	GGTGAGCCTCATAACAGAAGCTTT
HIF-2α	GGTTCGGGAGCACACTGTAT	CCTTCCTTCACAGAGCCAAG
IL-1β	TGCAGAGTTCCCCAACTGGTACATC	GTGCTGCCTAATGTCCCCTTGAATC
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
iNOS	AATCTTGGAGCGAGTTGTGG	CAGGAAGTAGGTGAGGGCTT
mCAD	AGGTTTCAAGATCGCAATGG	CTCCTTGGTGCTCCACTAGC
MGL1	ATGATGTCTGCCAGAGAACC	ATCACAGATTTCAGCAACCTTA
MRC1	TACAGCTCCACGCTATGGATT	CACTCTCCCAGTTGAGGTACT
PAI1	TCAGCCCTTGCTTGCCTCAT	GCATAGCCAGCACCGAGGA
PGC1α	CCTCCTCATAAAGCCAACCA	GGGCCGTTTAGTCTTCCTTT
PGK1	ATTCTGCTTGGACAATGGAGC	AGGCATGGGAACACCATCA
SAA	AGCGATGCCAGAGAGGCTGT	ACCCAGTAGTTGCTCCTCTT
SCD1	TGGGTTGGCTGCTTGTG	GCGTGGGCAGGATGAAG
TNFα	CGGAGTCCGGGCAGGT	GCTGGGTAGAGAATGGATCA
VEGFα	GGAGATCCTTCGAGGAGCACTT	GGCGATTTAGCAGCAGATATAAGAA

Blots were blocked with 5% nonfat milk and probed with primary antibodies, HIF-2α (Novus), HIF-1α and ARG1 (ABcam, MA), iNOS (Santa Cruz Biotechnology, CA) and β-actin (Sigma-Aldrich). The blots were visualized with horseradish peroxidase (HRP)–conjugated secondary antibodies (Sigma-Aldrich) and developed with chemiluminescence (ECL). For the detection of insulin signaling in 3T3-L1 adipocytes co-cultured with macrophages, 3T3-L1 cells were incubated in low-glucose DMEM containing 0.1% BSA for 8 hrs and then lysed after 30 min with 50nM insulin stimulation. For insulin signaling, antibodies against pAkt (Ser473), Akt, and pGSK (Ser9) (Cell Signaling, MA) and GSK (BD bioscience) were purchased.

Measurement of nitric oxide level. Nitrite was measured using the Griess reaction as described previously (Park et al., 2006). Culture media (100 μl) were collected and incubated with an equal volume of Griess reagent (Sigma-Aldrich) for 10 min at room temperature. The nitrite concentration was determined by the absorbance at 550 nm, using sodium nitrite as a standard. BEC (S-(2-boronoethyl)-L-cysteine) as an arginase inhibitor was purchased from Cayman Chemical, MI.

Statistical analysis. Results represent data from multiple, at least more than two times, independent experiments. Error bars represent standard deviation, and *P* values are calculated from the Student's t-test or two-way ANOVA.

Results

HIF-2α is increased in adipose tissue macrophages of obese mice

It has been shown that the expression levels of HIF-1α mRNA and protein are increased in obese adipose tissue (Hosogai et al., 2007; Ye et al., 2007). However, it is unclear whether the level of HIF-2α would be altered in adipose tissue of obese animals even though HIF-2α is another major transcription factor to mediate hypoxia. In order to address this, I quantitatively analyzed the level of HIF-2α mRNA in epididymal adipose tissue (EAT) of *db/db* mice. Similar to HIF-1α mRNA, the level of HIF-2α mRNA was significantly increased in *db/db* mice (Fig. 18A). Compared to lean mice, HIF-2α mRNA was elevated in both stromal vascular cells (SVCs) and adipocyte fraction from EAT of *db/db* mice (Fig. 18B). To test whether obese adipose tissue would indeed exhibit HIF-2α protein accumulation in ATMs, whole-mount immunofluorescence was performed. As shown in Fig.1A and B, HIF-2α protein were enhanced and co-localized with CD11b-positive macrophages in adipose tissue of *db/db* mice (Fig. 19). Next, I isolated M1-like ATMs ($CD11b^{+}F4/80^{+}CD11c^{+}$) and M2-like ATMs ($CD11b^{+}F4/80^{+}CD11c^{-}$) from SVCs of adipose tissue using flow cytometry and then examined the level of HIF-2α mRNA from M1 and M2 ATM-enriched populations. Compared with M1-like ATMs, the HIF-2α mRNA was highly expressed in M2-like ATMs along with M2 markers such as macrophage galactose N-acetyl-galactosamine-specific lectin 1 (MGL1), macrophage mannose receptor 1 (MRC1), and chitinase-3-like protein 3

Figure 18. HIF-2α is increased in adipose tissue from *db/db* mice. A: mRNA expression of HIF-2α, HIF-1α, hypoxia-response genes (GLUT1 and phosphoglycerate kinase 1; PGK1), adipocyte marker (adiponectin), and macrophage marker (F4/80) in epididymal adipose tissues (EATs) of db/db mice. B: Expression level of HIF-2α, HIF-1α, GLUT1, adiponectin, and F4/80 in adipocytes and stromal-vascular cells fraction from EATs of db/db mice. mRNA expression levels were analyzed using qRT-PCR and normalized by the level of cyclophilin mRNA. Data represent mean ± SD. $^{*}P < 0.05$, and $^{**}P < 0.01$ vs. heterozygote mice control; $^{\#}P < 0.05$ vs. adipocyte fraction by Student's *t*-test.

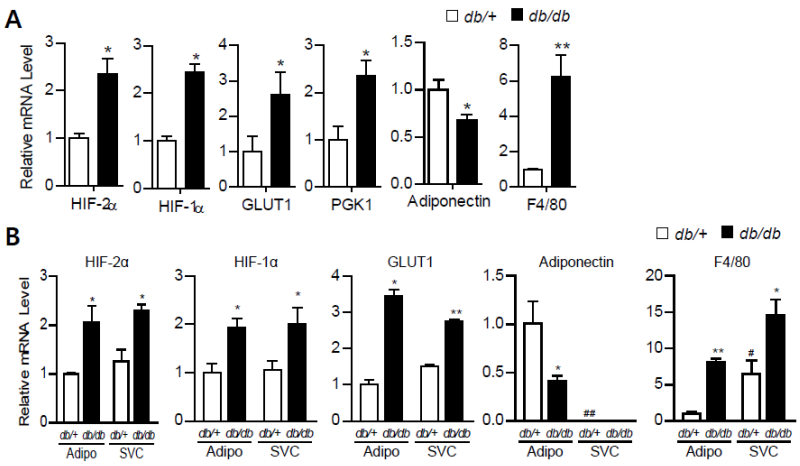

A
□ db/+ ■ db/db
Relative mRNA Level
HIF-2α
HIF-1α
GLUT1
PGK1
Adiponectin
F4/80
B
□ db/+ ■ db/db
Relative mRNA Level
HIF-2α
HIF-1α
GLUT1
Adiponectin
F4/80
db/+ db/db
Adipo
SVC

Figure 19. HIF-2α is elevated in ATMs from db/db mice. A-B: Expression pattern of HIF-2α in adipose tissue of lean db/+ and obese db/db mice. Whole-mount immunofluorescence analysis for the nucleus (blue), perilipin (green), CD11b (yellow) and HIF-2α (red) was performed on the epididymal adipose tissues (EATs) of db/+ and db/db mice. CLS, crown-like structure; A, adipocyte; M, macrophage.

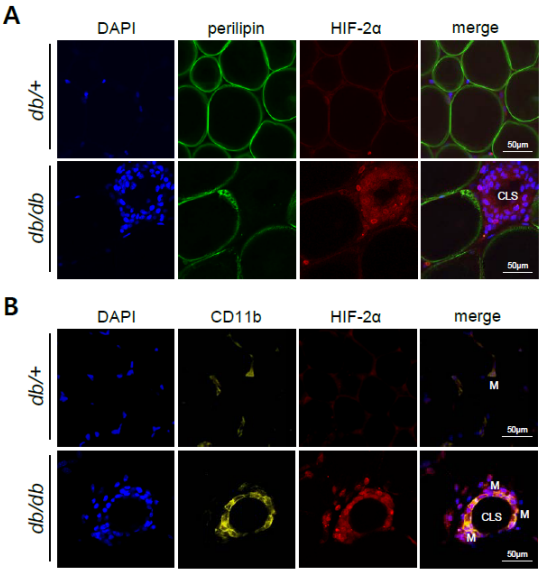
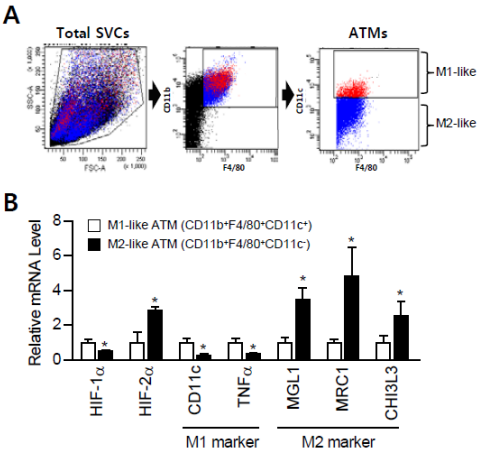

A
DAPI
perilipin
HIF-2α
merge
db/+
db/db
CLS
50μm
B
DAPI
CD11b
HIF-2α
merge
db/+
db/db
M
CLS
50μm

Figure 20. HIF-2α is abundant in M2-like ATMs compared to M1-like ATMs from db/db mice. A: Sorting M1-like and M2-like ATMs from the epididymal adipose tissues (EATs). EATs from db/db mice were dissected and fractionated into SVCs. SVCs were stained with antibodies against F4/80, CD11b, and CD11c and sorted into CD11c-positive and negative macrophages ($CD11b^+$ and $F4/80^+$ respectively) using the fluorescence-activated cell sorting (FACS) B: mRNA expression levels of HIF-2α, HIF-1α, and M1 and M2 markers in M1-like ATMs or M2-like ATMs from the epididymal adipose tissues (EATs) of db/db mice were determined using qRT-PCR. The gene expression levels were normalized by the level of cyclophilin mRNA. Data represent mean ± SD. *$P < 0.05$ M1-like vs. M2-like by Student's *t*-test.


A
Total SVCs
ATMs
M1-like
M2-like
CD11b
F4/80
CD11c
F4/80
B
M1-like ATM (CD11b+F4/80+CD11c+)
M2-like ATM (CD11b+F4/80+CD11c-)
Relative mRNA Level
0
2
4
6
8
HIF-1α
HIF-2α
CD11c
TNFα
MGL1
MRC1
CHI3L3
M1 marker
M2 marker

(CHI3L3) (Fig. 20). In contrast, HIF-1α mRNA was abundantly expressed in M1-like ATMs of obese animals.

Overexpression of macrophage HIF-2α diminishes pro-inflammatory responses via ARG1

It has been recently demonstrated that HIF-1α and HIF-2α would counteract to regulate NO synthesis, which partly affects macrophage polarity (Takeda et al., 2010). To examine the effect of HIF-2α elevation on pro-inflammatory activity of macrophages, I analyzed the gene expressions linked with NO metabolism and several inflammatory cytokines. In peritoneal macrophages infected with HIF-2α adenovirus (Ad-HIF-2α), HIF-2α overexpression stimulated the levels of ARG1 mRNA and protein, accompanied with angiogenic factors such as VEGFα and ADM (Fig. 21A~C). However, mRNA level of ARG2 and inducible nitric oxide synthase (iNOS) were not altered by HIF-2α overexpression (Fig. 21B). ARG1 competitively utilizes L-arginine, which is also a substrates of iNOS for NO synthesis, and actively prevents NO production in macrophages (Munder, 2009). In accordance with the gene expression profiles, NO production was significantly suppressed by HIF-2α overexpression in macrophages (Fig. 21D). On the contrary, treatment of ARG inhibitor, BEC, reversed the reduction of NO generation upon HIF-2α overexpression (Fig. 21E). Moreover, macrophage HIF-2α overexpression decreased the expression levels of pro-inflammatory cytokines such as TNFα, IL-6, and IL-1β, while BEC, reversed the inhibitory effect of HIF-2α on expression of

pro-inflammatory cytokine genes in macrophages (Fig. 21F). Interestingly, ARG1 induction by HIF-2α was not observed in 3T3-L1 preadipocytes or mature adipocytes unlike the mouse macrophage-like cell line, RAW264.7 (Fig. 22).

Because macrophages sensitively change their functional phenotypes in response to local microenvironmental inputs, GFP adenovirus (Ad-GFP)- or Ad-HIF-2α-infected macrophages were co-cultured with freshly isolated adipose tissues (Fig. 23A). As shown Fig. 23B, certain signaling molecules released from adipose tissues potently stimulated the expression of TNFα in co-cultured macrophages, whereas the induction of TNFα was greatly abolished by HIF-2α overexpression in macrophages. Interestingly, ARG1 mRNA was greatly induced in macrophages co-cultured with adipose tissue, whereas macrophage HIF-2α overexpression further augmented ARG1 mRNA (Fig. 23B). Together, these results suggest that macrophage HIF-2α could affect inflammatory response of ATMs through induction of ARG1, which might be influenced by signal molecules form adipose tissue.

Macrophage HIF-2α relives pro-inflammatory responses and insulin resistance in adipocytes

To explore the role of macrophage HIF-2α during the interaction with adipocytes, differentiated 3T3-L1 adipocytes were indirectly co-cultured with macrophages with or without HIF-2α overexpression (Fig. 24A). Adipocytes co-cultured with macrophages increased the mRNA level of pro-inflammatory genes such as iNOS, IL-6, and serum amyloid A (SAA), which were significantly reduced

Figure 21. Macrophage HIF-2α overexpression suppresses NO production and expression of inflammatory cytokine genes via induction of ARG1. A-E: Regulation of NO metabolism by HIF-2α overexpression in macrophages. Peritoneal macrophages were infected with adenoviral HIF-2α (Ad-HIF-2α) or adenoviral GFP (Ad-GFP). Total RNA was isolated and analyzed for HIF-2α, pro-angigogenic genes (VEGFα, ADM) (A), and NO metabolic genes (ARG1, ARG2, and iNOS) (B). Data represent mean ± SD. $^{**}P < 0.01$ vs. Ad-GFP control by Student's *t*-test. Total lysates were subjected to western blot analysis using specific antibodies (HIF-2α, ARG1, iNOS and β-actin). β-actin was used as the loading control (C). Ad-GFP- or Ad-HIF-2α-infected macrophages were treated with or without L-arginine (0.1~2 mM) (D) or BEC (0.1 mM) (E). After 48hr, cell culture media from these cells were harvested and measured NO concentration. F: Regulation of inflammatory cytokines by HIF-2α overexpression in macrophages. Ad-GFP- or Ad-HIF-2α-infected macrophages were treated with or without BEC (0.1 mM). After 48hr, mRNA expression levels of pro-inflammatory genes (TNFα, IL6, and IL1β). The gene expression levels were analyzed using qRT-PCR and normalized by the level of cyclophilin mRNA. Data represent mean ± SD. $^{*}P < 0.05$, and $^{**}P < 0.01$ vs. Ad-GFP control; $^{\#}P < 0.05$ vs. Ad-HIF-2α control by Student's *t*-test. $^{\S\S\S}P < 0.005$ vs. Ad-GFP control by two-way ANOVA.

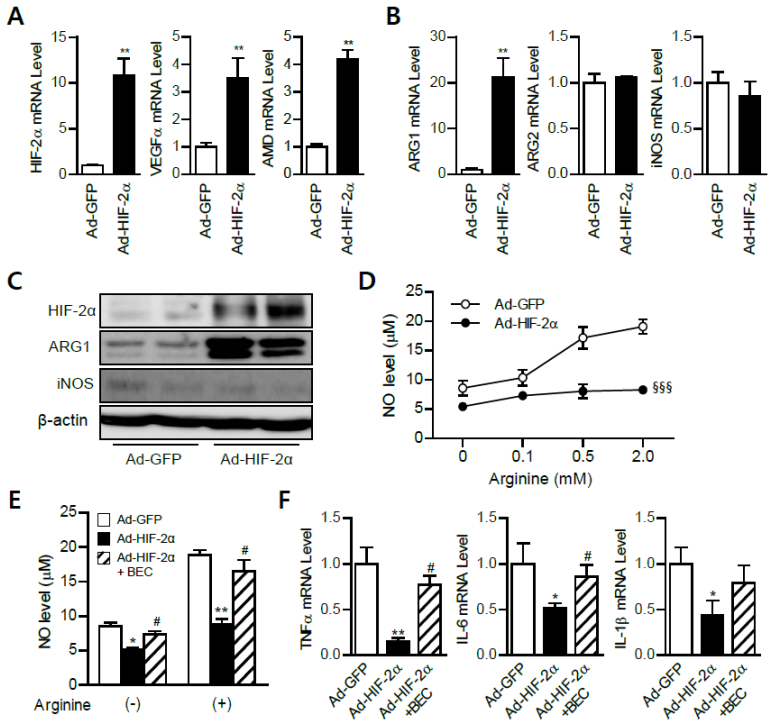

A
HIF-2α mRNA Level
VEGFα mRNA Level
AMD mRNA Level
Ad-GFP
Ad-HIF-2α
B
ARG1 mRNA Level
ARG2 mRNA Level
iNOS mRNA Level
C
HIF-2α
ARG1
iNOS
β-actin
Ad-GFP
Ad-HIF-2α
D
NO level (μM)
Ad-GFP
Ad-HIF-2α
§§§
Arginine (mM)
E
Ad-GFP
Ad-HIF-2α
Ad-HIF-2α + BEC
NO level (μM)
Arginine
(-)
(+)
F
TNFα mRNA Level
IL-6 mRNA Level
IL-1β mRNA Level
Ad-GFP
Ad-HIF-2α
Ad-HIF-2α +BEC

Figure 22. HIF-2α overexpression induces ARG1 mRNA in macrophages but not in adipocytes or pre-adipocytes. A-C: After infection of Ad-HIF-2α or Ad-GFP, total RNA was isolated and analyzed for HIF-2α and NO metabolic genes (ARG1, ARG2, and iNOS) in macrophage-like Raw264.7 cells (A), 3T3-L1 preadipocytes (B), and fully-differentiated 3T3-L1 adipocytes (C). The gene expression levels were analyzed using qRT-PCR and normalized by the level of cyclophilin mRNA. Data represent mean ± SD. *$P < 0.05$, and **$P < 0.01$ vs. Ad-GFP control by Student's *t*-test.

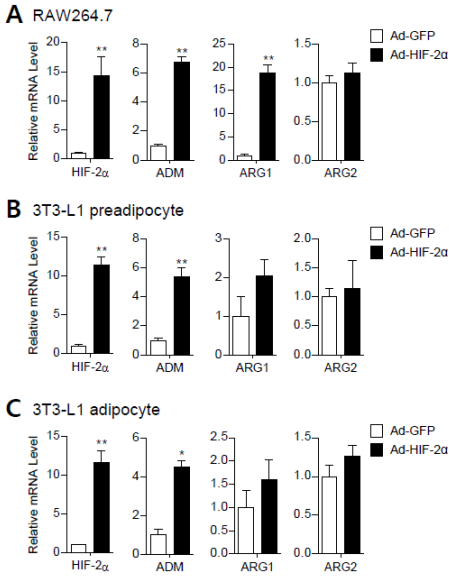
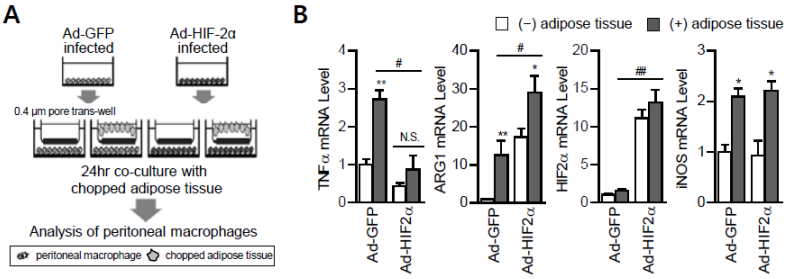

A RAW264.7
Relative mRNA Level
HIF-2α
ADM
ARG1
ARG2
Ad-GFP
Ad-HIF-2α
B 3T3-L1 preadipocyte
C 3T3-L1 adipocyte

Figure 23. HIF-2α overexpression suppresses TNFα mRNA, while further augments ARG1 mRNA in macrophages co-cultured adipose tissues. A-B: Ad-GFP- or Ad-HIF-2α-infected macrophages were indirectly co-cultured with chopped adipose tissues separating with transwell (0.4 μm pore) for 48hrs (A). Total RNA was isolated from macrophages and then analyzed for HIF-2α, ARG1, iNOS, and TNFα (B). Data represent mean ± SD. $*P < 0.05$, and $**P < 0.01$ vs. no co-culture control; $^{\#}P < 0.05$ vs. Ad-GFP control by Student's *t*-test. All qRT-PCR data were normalized by the level of cyclophilin mRNA.

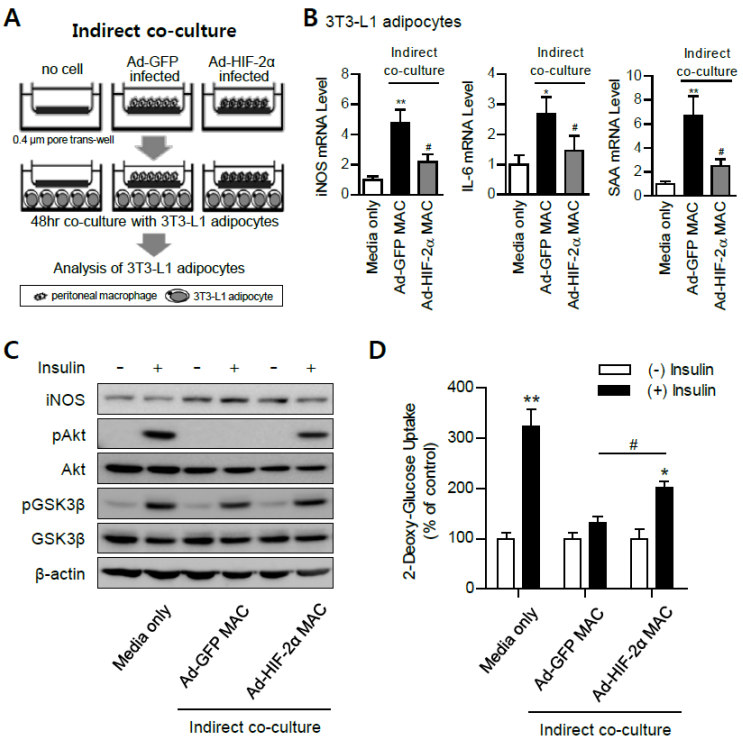

A
Ad-GFP infected
Ad-HIF-2α infected
0.4 μm pore trans-well
24hr co-culture with chopped adipose tissue
Analysis of peritoneal macrophages
peritoneal macrophage
chopped adipose tissue
B
(−) adipose tissue
(+) adipose tissue
TNFα mRNA Level
ARG1 mRNA Level
HIF2α mRNA Level
iNOS mRNA Level
Ad-GFP
Ad-HIF2α
N.S.

by co-culturing with HIF-2α-overexpressing macrophages (Fig. 24B). Compared to control adipocytes, adipocytes co-cultured with macrophages markedly inhibited the phosphorylation levels of Akt and GSK3β in the present of insulin, whereas adipocytes co-cultured with HIF-2α-overexpressing macrophages reversed the inhibitory effect of macrophages on insulin signaling in adipocytes (Fig. 24C). Accordingly, macrophages overexpressing HIF-2α restored insulin-stimulated glucose uptake ability of adipocytes, which was disrupted by co-culturing with macrophages (Fig. 24D). These data indicate that activation of macrophage HIF-2α would suppress pro-inflammatory responses and insulin resistance in adipocytes.

Macrophage HIF-2α knock-down accelerates palmitate-induced pro-inflammatory responses in adipocytes

To confirm the effects of macrophage HIF-2α on pro-inflammatory gene expression in adipocytes, I suppressed macrophage HIF-2α expression using siRNA. In macrophages, suppression of HIF-2α via siRNA decreased the expression of ARG1 mRNA (Fig. 25A). Nevertheless, in macrophages, the down-regulation of ARG1 by HIF-2α siRNA did not affect basal NO production in macrophages (Fig. 25B), probably, because NO generation is tightly restrained without induction of iNOS by certain stimuli. However, HIF-2α suppression significantly increased NO production upon palmitate challenge as one of metabolic stress conditions in macrophages (Fig. 25B), indicating that HIF-2α-suppressing macrophages could not maintain their ability to repress palmitate-induced NO production. In macrophages,

Figure 24. Macrophage HIF-2α overexpression relieves pro-inflammatory gene expression and insulin resistance in co-cultured adipocytes. A: Illustation of indirect co-culture system with adipocytes and macrophages. Peritoneal macrophages were seeded on the upper chamber of trans-well (0.4 μm pore) and infected with or without Ad-GFP or Ad-HIF-2α. 3T3-L1 adipocytes were differentiated on the lower chamber of trans-well and then co-cultured with prepared macrophages. After 48 hrs, adipocytes were harvested and used to extract total RNA. B: The relative mRNA levels of inflammatory genes (iNOS, IL6, and SAA) were analyzed by qRT-PCR. The gene expression levels were normalized by the level of cyclophilin mRNA. Data represent mean ± SD. $*P < 0.05$, and $**P < 0.01$ vs. no co-culture control; $^{\#}P < 0.05$ vs. Ad-GFP control by Student's *t*-test. C-D: Insulin signaling cascade and glucose uptake activity in adipocytes after co-culturing with or without Ad-GFP- or Ad-HIF-2α-infected macrophage. (C) After stimulation (30 min) with insulin (50 nM), adipocytes were harvested and total lysates were blotted with antibodies to detect iNOS and insulin signaling cascade (pAkt, total Akt, pGSK3β, pGSK3β). β-actin was used as the loading control. (D) Insulin dependent glucose uptake assays were performed as decribed in Materials and Methods. Relative folds of glucose uptake were shown in graph. Data represent mean ± SD. $*P < 0.05$, and $**P < 0.05$ vs. no treat of insulin control; $^{\#}P < 0.05$ vs. Ad-GFP-infected macrophage co-culture control by Student's *t*-test. Media only; adipocytes were cultured without macrophages co-culture, Ad-GFP MAC; adipocytes were co-cultured with Ad-GFP-infected macrophages, Ad-HIF-2α MAC; adipocytes co-cultured with Ad- HIF-2α-infected macrophages.

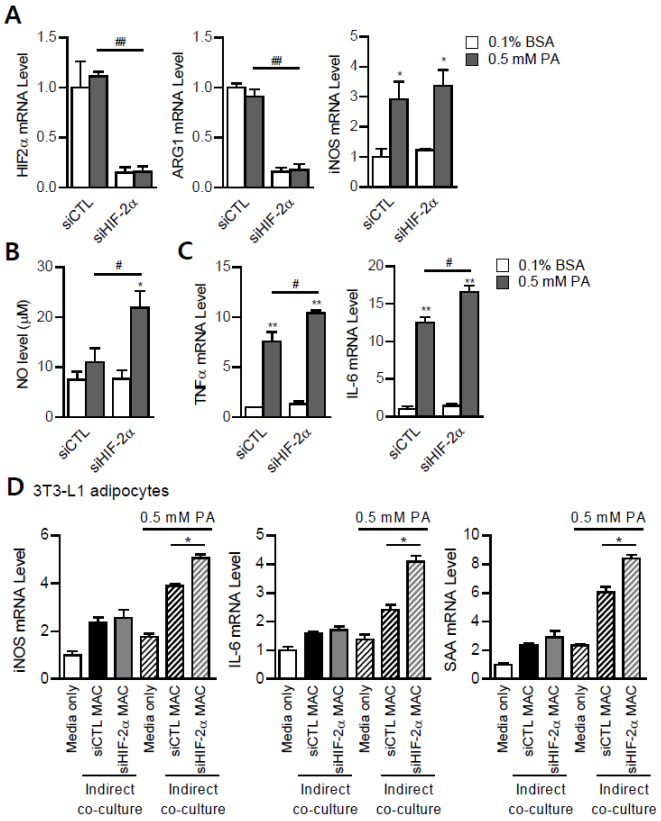

A
Indirect co-culture
no cell
Ad-GFP infected
Ad-HIF-2α infected
0.4 μm pore trans-well
48hr co-culture with 3T3-L1 adipocytes
Analysis of 3T3-L1 adipocytes
peritoneal macrophage
3T3-L1 adipocyte
B 3T3-L1 adipocytes
Indirect co-culture
iNOS mRNA Level
IL-6 mRNA Level
SAA mRNA Level
Media only
Ad-GFP MAC
Ad-HIF-2α MAC
C
Insulin
iNOS
pAkt
Akt
pGSK3β
GSK3β
β-actin
Media only
Ad-GFP MAC
Ad-HIF-2α MAC
Indirect co-culture
D
(-) Insulin
(+) Insulin
2-Deoxy-Glucose Uptake (% of control)
Media only
Ad-GFP MAC
Ad-HIF-2α MAC
Indirect co-culture

HIF-2α suppression further promoted the mRNA levels of TNFα and IL-6, which are key mediators of pro-inflammatory responses in adipose tissue (Fig. 25C). Furthermore, when differentiated 3T3-L1 adipocytes were indirectly co-cultured with macrophages in the presence or absence of HIF-2α siRNA, macrophage HIF-2α suppression elevated mRNA levels of pro-inflammatory genes such as iNOS, IL-6, and SAA in adipocytes upon palmitate (Fig. 25D). These results clearly suggest that macrophage HIF-2α may attenuate pro-inflammatory responses, which would be aggravated by the active cross-talk between adipocytes and recruited macrophages upon metabolic stresses such as hyperlipidemic condition in obesity.

HIF-2α$^{+/-}$ mice exhibit insulin resistance upon HFD

To further investigate *in vivo* role of HIF-2α in adipose tissue inflammation and insulin resistance, I decided to examine HIF-2α knock-out mice model. Since HIF-2α whole body knock-out mice are embryonic lethal (Tian et al., 1998), HIF-2α haplodeficient mice (HIF-2α$^{+/-}$) were fed either normal chow diet (ND) or HFD for 16 weeks and compared with wild type (HIF-2α$^{+/+}$) of their littermates as a control group. As shown in Fig. 26, body weight, adipose tissue weight, adipocytes size, and serum triglycerides and cholesterol were not different between WT and HIF-2α$^{+/-}$ mice fed either ND or HFD. Also, there were no significant differences in gene expression profiles for lipid metabolism between WT and HIF-2α$^{+/-}$ mice (Fig. 27). However, HFD-fed HIF-2α$^{+/-}$ mice showed higher fasting insulin levels than HFD-fed WT mice with tendency of elevated fating glucose level (Fig. 28A and B). To

Figure 25. Macrophage HIF-2α knock-down promotes inflammatory response in adipocytes. A-C: Macrophages were transfected with control siRNA (siCTL) or HIF-2α specific siRNA (siHIF-2α) and then treated with 0.5 mM palmitate (PA) in 0.1% BSA or only 0.1% BSA. After 48 hrs, total RNA was extracted and analyzed by qRT-PCR for HIF-2α, ARG1, iNOS (A), and TNFα, IL6 (C). Cultured media were used for measurement of NO concentration (B). D: HIF-2α knock-down macrophages were indirectly co-cultured with adipocytes similar as illustrated in Fig. 3A. During co-culture (48hr), both adipocytes and macrophages were treated with or without 0.5 mM PA. The relative mRNA levels of iNOS, IL6 and SAA were analyzed by qRT-PCR. Data represent mean ± SD. $^{*}P < 0.05$, and $^{**}P < 0.01$ vs. BSA control; $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ vs. control siRNA by Student's *t*-test. mRNA expression levels were normalized by the level of cyclophilin mRNA. Media only; adipocytes were cultured without macrophages, siCTL MAC; adipocytes co-cultured with control siRNA-transfected macrophages, siHIF-2α MAC; adipocytes co-cultured with HIF-2α siRNA-transfected macrophages.


A
HIF2α mRNA Level
ARG1 mRNA Level
iNOS mRNA Level
0.1% BSA
0.5 mM PA
siCTL
siHIF-2α
B
NO level (μM)
C
TNFα mRNA Level
IL-6 mRNA Level
D
3T3-L1 adipocytes
0.5 mM PA
iNOS mRNA Level
IL-6 mRNA Level
SAA mRNA Level
Media only
siCTL MAC
siHIF-2α MAC
Indirect co-culture

explore whether haplodeficiency of HIF-2α would affect systemic insulin sensitivity, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were carried out. Similar to fasting insulin and glucose levels, there were no significant difference in OGTT and ITT between WT and HIF-2α$^{+/-}$ mice under ND feeding. However, it is of interest to note that HIF-2α$^{+/-}$ mice were more glucose and insulin intolerant than WT mice upon HFD (Fig. 28C and D). These observations evidently indicate that HIF-2α haplodeficiency is associated with systemic insulin resistance in diet-induced obesity (DIO).

In DIO, HIF-2α$^{+/-}$ mice aggravate adipose tissue inflammation with increased ATM accumulation

To determine whether accelerated insulin resistance is coupled with adipose tissue inflammation in HIF-2α$^{+/-}$ mice, I examined inflammatory gene expression and macrophage infiltration. In adipose tissues from HFD-fed mice, haplodeficiency of HIF-2α decreased the level of ARG1 expression whereas iNOS mRNA and protein slightly but substantially increased (Fig. 29A~C), which appeared to be consistant with the data from various *in vitro* cell culture and *ex vivo* experiments (Fig. 21~25). In addition, the expression of pro-inflammatory genes including TNFα, IL-6, and SAA was enhanced in adipose tissue of HFD-fed HIF-2α$^{+/-}$ mice (Fig. 29D). I also observed that HIF-2α$^{+/-}$ mice exhibited more numbers of crown-like structures (CLSs), consisting mostly of CD11c-positive ATMs, than WT mice upon HFD (Fig. 30A and B) In accordance with these, the numbers of

Figure 26. HIF-2$\alpha^{+/-}$ mice exhibit to increase body weight and fat weight upon HFD similar to WT mice. Ten-week-old HIF-2$\alpha^{+/-}$ mice and their WT littermates (n = 7~9 per group) were fed ND or HFD for 16 weeks. A: Body weight was measured every 2 weeks. B-C: Body weight gain (B) and the weights of various tissues (C) from HIF-2$\alpha^{+/-}$ and WT mice were measured after 16 week feeding either ND or HFD. N, normal diet; H, high-fat diet; WAT, white adipose tissue; Sub, subcutaneous fat; Epi, epididymal fat; Peri, perirenal fat; BAT, brown adipose tissue. D: Average adipocyte size was quantified with section images from epididymal adipose tissues (EATs). E-H: Levels of triglyceride (E), total cholesterol (F) were measured with serum samples. Data represent mean ± SD. *$P < 0.05$ vs. WT group by Student's *t*-test.

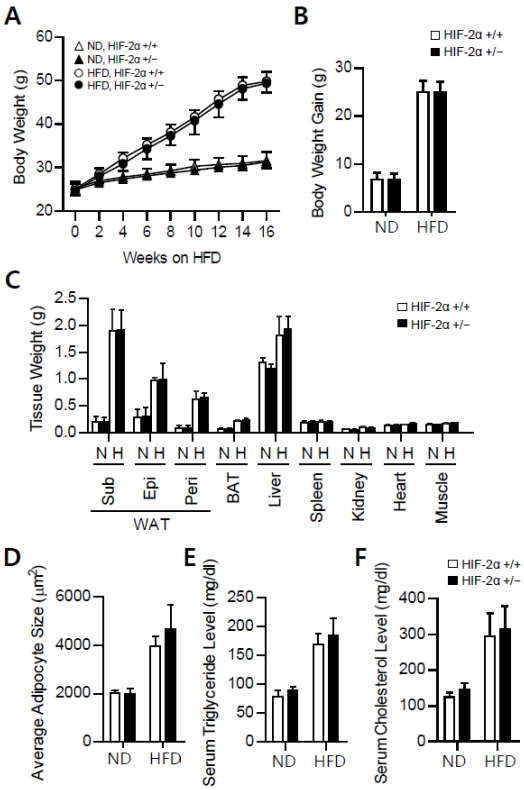

A
△ ND, HIF-2α +/+
▲ ND, HIF-2α +/−
○ HFD, HIF-2α +/+
● HFD, HIF-2α +/−
Body Weight (g)
Weeks on HFD
B
□ HIF-2α +/+
■ HIF-2α +/−
Body Weight Gain (g)
ND
HFD
C
□ HIF-2α +/+
■ HIF-2α +/−
Tissue Weight (g)
N H
Sub
Epi
Peri
WAT
BAT
Liver
Spleen
Kidney
Heart
Muscle
D
Average Adipocyte Size (μm²)
E
Serum Triglyceride Level (mg/dl)
F
Serum Cholesterol Level (mg/dl)

Figure 27. Gene expressions linked to lipid synthesis and oxidation is not different in adipose tissues of HIF-2α$^{+/-}$ and WT mice. A-B: Relative transcript levels of lipogenic genes (AC1, FAS, SCD1) (A) and lipid oxidative genes (PGC1α, ACO1, and mCAD) (B) in epididymal adipose tissues (EATs) were analyzed by qRT-PCR The gene expression levels were analyzed using qRT-PCR and normalized by the level of cyclophilin mRNA. Data represent mean ± SD. *P < 0.05 vs. WT group by Student's *t*-test.

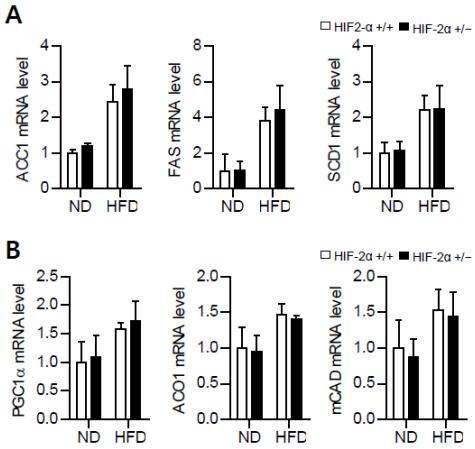
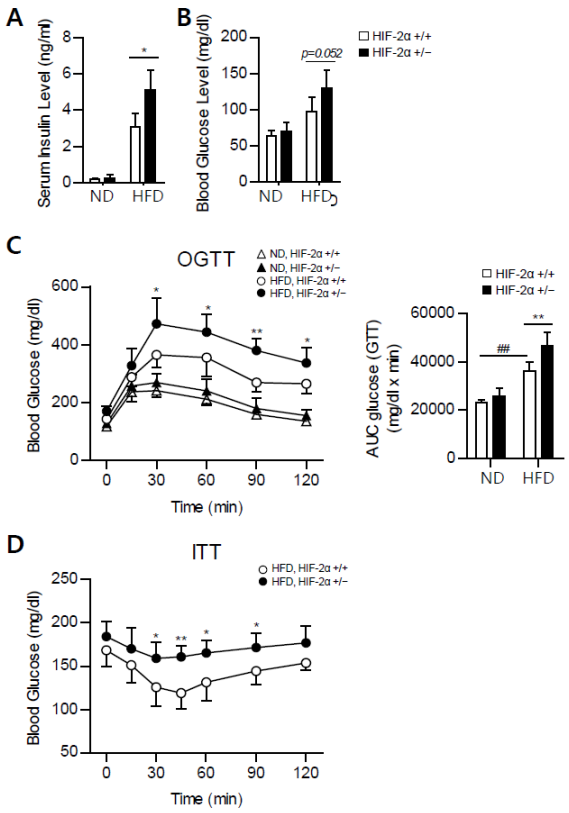

A
□ HIF2-α +/+ ■ HIF-2α +/−
ACC1 mRNA level
FAS mRNA level
SCD1 mRNA level
ND HFD
B
□ HIF-2α +/+ ■ HIF-2α +/−
PGC1α mRNA level
ACO1 mRNA level
mCAD mRNA level
ND HFD

Figure 28. HIF-2α$^{+/-}$ mice are more glucose intolerant upon HFD. A-B: In ND- or HFD-fed HIF-2α$^{+/-}$ and WT mice, levels of insulin (A) were measured with serum samples and level of fasting glucose (B) was measured with blood sample. C-D: Oral glucose tolerance test (OGTT) and area under curve (AUC) analysis (C) and Insulin tolerance test (ITT) (D) in HIF-2α$^{+/-}$ mice and WT mice. Data represent mean ± SD. **P* < 0.05, and ***P* < 0.01 vs. WT group; $^{\#\#}$*P* < 0.01 vs. ND-fed WT group by Student's *t*-test.


A
Serum Insulin Level (ng/ml)
8
6
4
2
0
*
ND
HFD
B
Blood Glucose Level (mg/dl)
200
150
100
50
0
p=0.052
ND
HFD
HIF-2α +/+
HIF-2α +/−
C
OGTT
ND, HIF-2α +/+
ND, HIF-2α +/−
HFD, HIF-2α +/+
HFD, HIF-2α +/−
Blood Glucose (mg/dl)
600
400
200
0
0
30
60
90
120
Time (min)
AUC glucose (GTT) (mg/dl x min)
60000
40000
20000
0
##
**
ND
HFD
D
ITT
HFD, HIF-2α +/+
HFD, HIF-2α +/−
Blood Glucose (mg/dl)
250
200
150
100
50
0
30
60
90
120
Time (min)

F4/80 and CD11b double-positive macrophages as well as M1-like ATMs per gram of fat mass were significantly promoted in adipose tissue of HFD-fed HIF-2α$^{+/-}$ mice, compared to HFD-fed WT mice (Fig. 30C). The percentages of F4/80, CD11b and CD11c triple-positive ATMs in SVCs were also elevated in adipose tissue of HIF-2α$^{+/-}$ mice, whereas the proportions of F4/80, CD11b positive, CD11c negative ATMs (containing M2-like macrophages) were not significantly changed in HIF-2α$^{+/-}$ mice (Fig. 30D and E). To explore the effect of HIF-2α on macrophage polarity, the expression levels of M1 and M2 marker genes were examined in adipose tissue of HFD-fed mice. As shown in Fig. 30F, in DIO, M1 marker genes such as F4/80 and CD11c were increased in HIF-2α$^{+/-}$ mice compared to WT mice, whereas M2 marker genes such as MGL1, MRC1, and CHI3L3 were not changed or slightly decreased. Taken together, these results strongly suggest that HIF-2α$^{+/-}$ mice are susceptible to adipose tissue inflammation with increased M1 ATMs in DIO.

In HIF-2α$^{+/-}$ mice, macrophage depletion improves insulin resistance and adipose tissue inflammation

To test the idea whether the increases in numbers and abundance of M1 ATMs might be essential for insulin resistance in HFD-fed HIF-2α$^{+/-}$ mice, I decided to deplete phagocytic macrophages using clodronate liposome (van Rooijen and Hendrikx, 2010). Expectedly, clodronate treatment significantly decreased the numbers of CLSs in adipose tissues from both HFD-fed HIF-2α$^{+/-}$ and HFD-fed WT

Figure 29. HIF-2$\alpha^{+/-}$ mice are more susceptible to adipose tissue inflammation upon HFD. A: Protein level of HIF-2α in adipose tissue of HIF-2$\alpha^{+/-}$ and WT mice fed ND or HFD. Whole-mount immunofluorescence analysis was performed for the nucleus (blue), perilipin (green), and HIF-2α (red). B-D: Relative mRNA levels of HIF-2α, ARG1, and iNOS transcripts in epididymal adipose tissues (EATs) were analyzed by qRT-PCR (B) and total lysates were subjected to Western blot analysis with antibodies specific for HIF-2α, ARG1, iNOS, and β-actin (C). β-actin was used as the loading control. (D) Relative mRNA levels of inflammatory cytokine genes (TNFα, IL6, and SAA) in EATs were analyzed by qRT-PCR. mRNA expression levels were normalized by the level of cyclophilin mRNA. Data represent mean ± SD. *P < 0.05, and **P < 0.01 vs. WT group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, and $^{\#\#\#}P$ < 0.001 vs. ND group by Student's *t*-test.

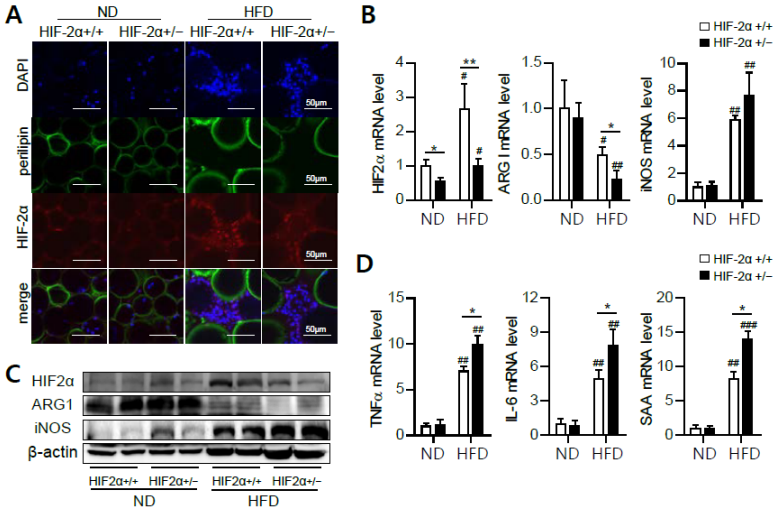
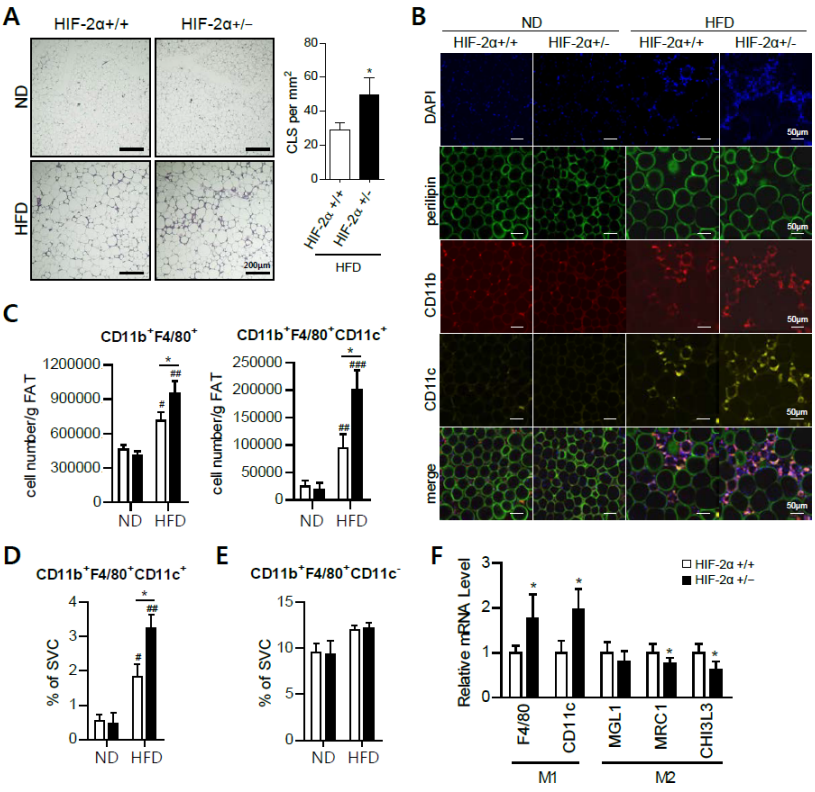

A
ND
HFD
HIF-2α+/+
HIF-2α+/−
HIF-2α+/+
HIF-2α+/−
DAPI
perilipin
HIF-2α
merge
50μm
B
HIF-2α +/+
HIF-2α +/−
HIF2α mRNA level
ARG I mRNA level
iNOS mRNA level
ND
HFD
C
HIF2α
ARG1
iNOS
β-actin
HIF2α+/+
HIF2α+/−
HIF2α+/+
HIF2α+/−
ND
HFD
D
TNFα mRNA level
IL-6 mRNA level
SAA mRNA level

Figure 30. M1-like macrophage population was elevated in adipose tissue of HIF-2α$^{+/-}$ mice upon HFD. A: Histological analysis was performed with epididymal adipose tissues (EATs) from HIF-2α$^{+/-}$ and WT mice and the numbers of CLSs were quantified. B: To confirm the phenotype of ATMs in, whole-mount immunofluorescence analysis for the nucleus (blue), perilipin (green), CD11b (red) and CD11c (yellow) was performed on the EATs of HIF-2α$^{+/-}$ and WT mice fed ND or HFD. C-D: Macrophage infiltration and M1 polarization were measured in EATs using flow cytometry analysis. (C) Total number of macrophages (double positive; CD11b^{+}, F4/80^{+}) and M1-like macrophages (triple positive; CD11b^{+}, F4/80^{+}, CD11c^{+}) among SVCs per gram of fat mass; (D) Percentages of CD11c-positieve and (E) CD11c-negative macrophage among SVCs in adipose tissue; F: Macrophage polarization marker genes were analyzed by qRT-PCR with total RNA extracted from SVCs fractionated in EATs from HFD-fed mice. Data represent mean ± SD. *P < 0.05, and **P < 0.01 vs. WT group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, and $^{\#\#\#}P$ < 0.001 vs. ND group by Student's t-test. mRNA expression levels were normalized by the level of cyclophilin mRNA.


A
HIF-2α+/+
HIF-2α+/−
ND
HFD
200μm
CLS per mm2
HIF-2α +/+
HIF-2α +/-
HFD
B
ND
HFD
HIF-2α+/+
HIF-2α+/-
DAPI
perilipin
CD11b
CD11c
merge
50μm
C
CD11b+F4/80+
CD11b+F4/80+CD11c+
cell number/g FAT
ND
HFD
D
CD11b+F4/80+CD11c+
% of SVC
E
CD11b+F4/80+CD11c-
F
HIF-2α +/+
HIF-2α +/−
Relative mRNA Level
F4/80
CD11c
MGL1
MRC1
CHI3L3
M1
M2

mice without changes in adipocyte size or morphology (Fig. 31A~C). Consistently, the mRNA levels of F4/80 and CD11c were greatly decreased by clodronate (Fig. 31D), and thereby the difference of ATM contents between HIF-2α$^{+/-}$ and WT mice was insignificant. Interestingly, macrophage depletion also decreased the level of ARG1 mRNA, while the expression level of HIF-2α was not altered (Fig. 31E). These observation imply that expression of ARG1 might be more abundant in ATMs of adipose tissue while HIF-2α seems to be broadly expressed in various cell types including adipocytes, macrophages and endothelial cells. In spite of decreased ARG1 expression, mediating anti-inflammatory effects of macrophage HIF-2α, clodronate-mediated depletion of macrophages explicitly improved glucose intolerance in HFD-fed HIF-2α$^{+/-}$ mice, comparable to HFD-fed WT mice (Fig. 31F). Therefore, these data suggest that enhanced recruitment and pro-inflammatory polarization of ATMs would be closely associated with systemic insulin resistance in HFD-fed HIF-2α$^{+/-}$ mice.

Haploid deletion of HIF-2α did not affect angiogenesis in adipose tissue

Recent reports suggested that impaired vascularization in adipose tissue contributes to adipose tissue inflammation and insulin resistance in obesity (Sung et al., 2013; Yilmaz and Hotamisligil, 2013). Furthermore, because primary targets of HIF-2α are angiogenesis-related genes such as VEGFα and ADM, there is possibility that adipose tissue inflammation exhibited in HIF-2α$^{+/-}$ mice may be caused by insufficiency of vasculature. Thus, I investigated whether haploid

Figure 31. HIF-2α$^{+/-}$ mice exhibit improved glucose tolerance by macrophage depletion. A-C: Intraperitoneal injections of clodronate liposome were given to HIF-2α$^{+/-}$ and WT mice (n = 6 per group) fed HFD for 12weeks. Depletion of macrophages in epididymal adipose tissues (EATs) of HIF-2α$^{+/-}$ and WT mice by clodronate (Clo.) was analyzed by histological analysis (A) and qRT-PCR analysis for macrophage marker, F4/80 and CD11c (B). (C) HIF-2α and ARG1 mRNA were also analyzed in by qRT-PCR. D: OGTT were performed 6 days after the first injection of clodronate and PBS control. Data represent mean ± SD. *$P < 0.05$, and **$P < 0.01$ vs. WT group, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ vs. PBS-injected control mice group by Student's *t*-test. §§ $P < 0.001$ vs. WT mice group by two-way ANOVA. mRNA expression levels were normalized by the level of cyclophilin mRNA.

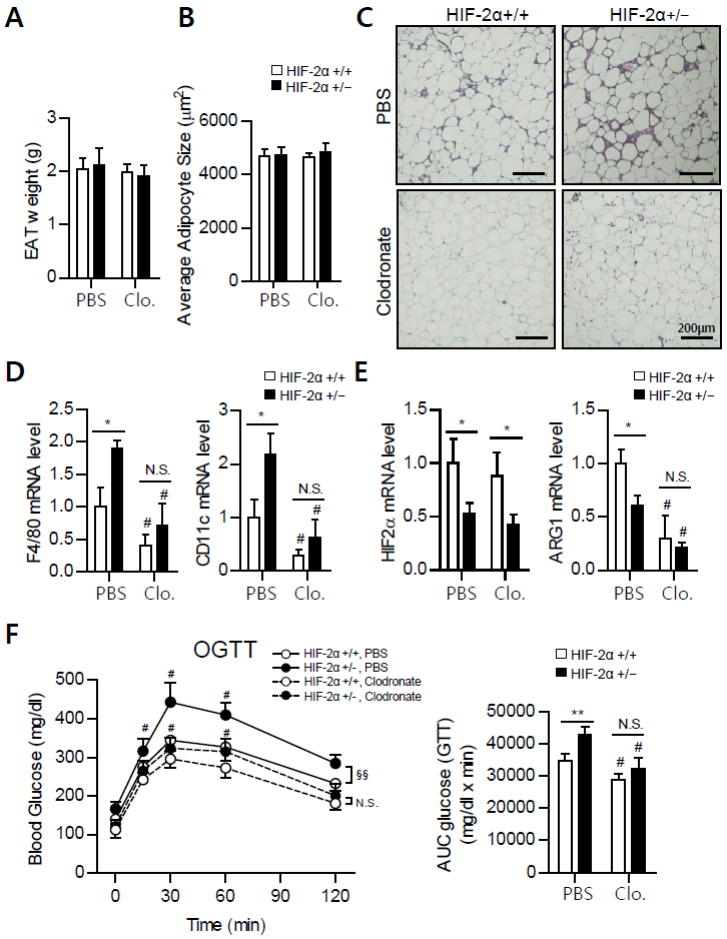
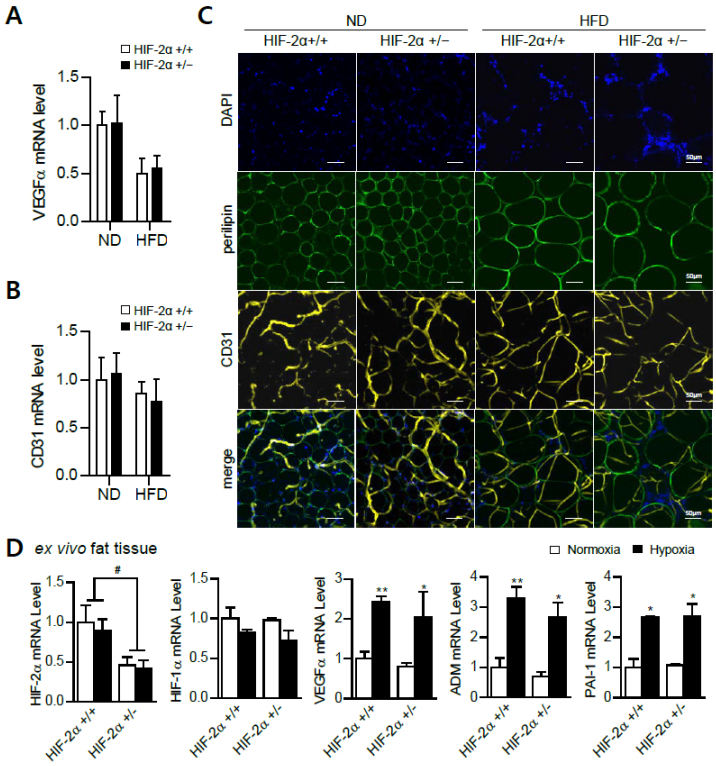

A
B
C
HIF-2α+/+
HIF-2α+/−
PBS
Clodronate
EAT weight (g)
Average Adipocyte Size (μm²)
HIF-2α +/+
HIF-2α +/−
PBS
Clo.
200μm
D
E
F
F4/80 mRNA level
CD11c mRNA level
HIF2α mRNA level
ARG1 mRNA level
N.S.
OGTT
HIF-2α +/+, PBS
HIF-2α +/- , PBS
HIF-2α +/+, Clodronate
HIF-2α +/- , Clodronate
Blood Glucose (mg/dl)
Time (min)
AUC glucose (GTT) (mg/dl x min)

Figure 32. Blood vessel density is not different between HIF-2α$^{+/-}$ and WT mice. A-B: Relative mRNA levels of VEGFα and CD31 in epididymal adipose tissues (EATs) were analyzed by qRT-PCR. C: Whole-mount immunofluorescence analysis for blood vessel density was performed on the EATs from HIF-2α$^{+/-}$ and WT mice fed ND or HFD for 16 weeks. CD31 (yellow) signal was accumulated 80 μm thickness. The nucleus (blue); Perilipin (green). D: Chopped adipose tissues were *ex vivo* cultured under normoxic (21% oxygen) or hypoxic (1% oxygen) condition for 24hrs. The expression levels of HIF family (HIF-1α and HIF-2α) and hypoxia-response genes (VEGFα, ADM, and PAI1) were analyzed by qRT-PCR.


A
HIF-2α +/+
HIF-2α +/−
VEGFα mRNA level
1.5
1.0
0.5
0.0
ND
HFD
B
HIF-2α +/+
HIF-2α +/−
CD31 mRNA level
1.5
1.0
0.5
0.0
ND
HFD
C
ND
HFD
HIF-2α+/+
HIF-2α +/−
HIF-2α+/+
HIF-2α +/−
DAPI
perilipin
CD31
merge
50μm
D
ex vivo fat tissue
Normoxia
Hypoxia
HIF-2α mRNA Level
HIF-1α mRNA Level
VEGFα mRNA Level
ADM mRNA Level
PAI-1 mRNA Level
#
**
*
HIF-2α +/+
HIF-2α +/-

deletion of HIF-2α affects vascularization in adipose tissue upon HFD. Although HFD have reduced VEGFα expression in adipose tissue, HIF-2α$^{+/-}$ mice did not have an additional reduction of VEGFα mRNA in adipose tissue (Fig. 32A). In addition, mRNA level of CD31, endothelial cell marker, also was the same in adipose tissue of HIF-2α$^{+/-}$ and WT mice (Fig. 32B). To compare vascular structure in adipose tissue of these mice via visualization, I performed whole-mount immunofluorescence against CD31 and get blood vessel images accumulating of adipose tissue (Fig. 32C). Consistent with mRNA data, there were no significant differences of vascular density in adipose tissue from both normal and HFD-fed mice. In fact, *ex vivo* culture with chopped adipose tissue from HIF-2α$^{+/-}$ and WT mice, angiogenesis-related genes such as VEGFα, ADM and PAI-1 clearly induced by hypoxia compare to normoixa (Fig. 32D). As a result, these data suggest that vasculature is not reduced in adipose tissue of HIF-2α$^{+/-}$ mice, implying that biological processes beyond angiogenesis such as macrophage physiology would be more responsible to adipose tissue inflammation and insulin resistance by HIF-2α haploinsufficiency.

Discussion

In obese adipose tissue, the decrease of vasculature network and blood flow reduces local oxygen tension, which leads to adipose tissue hypoxia (Sun et al., 2011). Emerging evidence has suggested that activation of HIF-1α in obesity mediates adipose tissue inflammation and macrophage infiltration as well as classical hypoxic responses such as angiogenesis and glycolysis (Fujisaka et al., 2009; Lumeng et al., 2007a; Sun et al., 2011). In obesity, adipose tissue hypoxia induces M1-like polarization in ATMs, where HIF-1α contributes to increase pro-inflammatory response in ATMs (Fujisaka et al., 2013). Despite of these findings, it is largely unknown which pathways are involved in anti-inflammatory responses or resolution mechanisms against excessive pro-inflammatory responses in ATMs of obesity. It has been reported that both M1- and M2-like ATMs are increased in adipose tissue of obesity (Fujisaka et al., 2009; Fujisaka et al., 2013; Odegaard and Chawla, 2011; Shaul et al., 2010). The recent finding that HIF-2α, another key player for hypoxic responses, has its own target genes and is induced by Th2 cytokines associated with M2 macrophage polarization (Takeda et al., 2010) led us to explore the functional role of HIF-2α in adipose tissue of obesity. Our data suggest that HIF-2α activation in ATMs, in opposite to HIF-1α, is involved in amelioration of adipose tissue inflammation and insulin resistance in obesity.

NO acts as a signaling molecule to mediate various physiological roles including vasodilator, neurotransmitter, and muscle relaxing (Adams et al., 1999). However, excessive NO generation by iNOS is well known to interfere insulin

signaling in obesity (Olefsky and Glass, 2010; Wellen and Hotamisligil, 2003, 2005). For example, iNOS knock-out mice are protected from HFD-induced insulin resistance whereas treatment of NO donor deteriorates insulin signaling and adipocytokines (Nozaki et al., 2007; Ovadia et al., 2011; Perreault and Marette, 2001). Recently, ARG1, highly expressed in M2 ATMs, has been suggested as an opposite regulator of iNOS activity by competitive utilization of a common precursor, L-arginine, in lean adipose tissue (Chawla et al., 2011; Odegaard and Chawla, 2011). It is not yet completely understood whether HIF-2α activation might drive M2 polarization in ATMs. In this study, our data suggest that activation of macrophage HIF-2α would stimulate ARG1 expression, which helps macrophages to maintain M2 type characters by reducing NO production and pro-inflammatory cytokine expression (Fig. 21). Furthermore, HIF-2α-overexpressing macrophages were able to suppress pro-inflammatory responses induced by adipocyte-derived inflammatory mediators including free fatty acids (Fig. 23 and 25). Given that ARG1 would be a key mediator for anti-inflammatory function of macrophage HIF-2α (Fig. 21), it is likely that anti-inflammatory features of macrophage HIF-2α would be, at least partly, mediated through reduction of reactive nitrogen species, which could readily react with reactive oxygen species to boost pro-inflammatory responses and insulin resistance. Accordingly, macrophage HIF-2α overexpression protected against inflammatory signals and insulin resistance driven by cross-talk between adipocytes and macrophage (Fig. 24). Thus, our data imply that macrophage HIF-2α activation would be one of resolving pathways to induce M2

activation in adipose tissue of obesity.

Although HIF-2α$^{+/-}$ mice did not show any distinct feature with WT mice without stresse conditions, several phenotypes have been reported upon specific stimuli inducing prolonged hypoxia (Brusselmans et al., 2003; Dioum et al., 2008; Peng et al., 2011). In addition, it has been demonstated that haplodeficiency of HIF-2α suppresses osteoarthritis developments independent of hypoxia (Saito et al., 2010; Yang et al., 2010). In the present study, we provide another character of HIF-2α$^{+/-}$ mice, who are susceptible to adipose tissue inflammation and insulin resistance in DIO. In adipose tissue of HIF-2α$^{+/-}$ mice, CD11c-positive M1-like macrophage population was greatly elevated upon HFD (Fig. 29). In DIO, both WT and HIF-2α$^{+/-}$ mice similarly increased body weight, adiposity and serum lipid metabolites except serum glucose and insulin. Furthermore, HFD-fed HIF-2α$^{+/-}$ mice were insulin resistant compared to HFD-fed WT mice. Moreover, given that macrophage depletion using clodronate improved insulin resistance in HFD-fed HIF-2α$^{+/-}$ mice, it is very likely that polarity change of ATMs by HIF-2α haplodeficiency would be critical for systemic insulin intolerance in HIF-2α$^{+/-}$ mice (Fig. 27~29). Therefore, these results lead us to consider macrophage HIF-2α as a key mediator of ant-inflammatory responses in adipose tissue in response to metabolic stresses. Nevertheless, it has been reported that HIF-2α is able to regulate GLUT1, GLUT4, IRS2, and IRS3 to promote insulin signaling (Shimba et al., 2004; Wei et al., 2013). Thus, it remains to be elucidated whether in HFD-fed HIF-2α$^{+/-}$ mice, deteriorated insulin resistance might be resulted from increase of

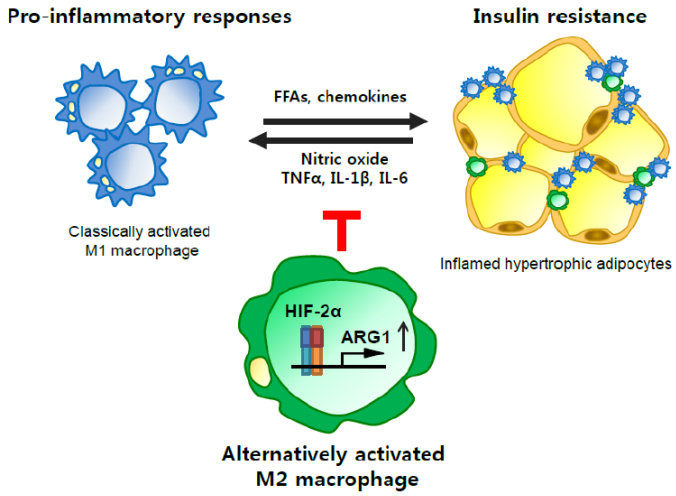
inflammatory response in HIF-2α insufficient ATMs.

In accordance with the findings that macrophage HIF-2α overexpression would decrease pro-inflammatory interplay between macrophages and adipocytes by induction of ARG1, we observed that HFD-fed HIF-2α$^{+/-}$ mice augmented pro-inflammatory responses in adipose tissue. However, our *in vivo* data revealed that HIF-2α accmulation in ATMs of obesity would not be sufficient to induce ARG1 to resolve pro-inflammatory responses (Fig. 28), implying that induction of ARG1 by HIF-2α *in vivo* might not be enough to overcome accumulating pro-inflammatory inputs in obese adipose tissue. Althoug it is unclear why and how elevated HIF-2α does not increase ARG1 in obese adipose tissue, there are several possibilites to explain the distinct expression profiles of HIF-2α and ARG1 *in vivo* and *in vitro*. One of possible mechanisms for incapability of increased macrophage HIF-2α to elevate ARG1 in obesity might be resulted from protein modifications. Although HIF-2α is primarily regulated by oxygen-dependent post-translational stabilization, its activity is influenced by various oxygen-independent modifications such as acetylation and phosphorylation (Keith et al., 2012). In particular, mammalian silent information regulator 2 homolog (SIRT1), which is involved in the regulation of adipose tissue inflammation as a key energy sensor, deacetylates HIF-2α. Compared to acetylated HIF-2α, SIRT1-dependent HIF-2α deaceylation stimulates transcriptional activity of HIF-2α (Cho and Lumeng, 2011; Dioum et al., 2009). It is well-established that, in DIO, reduction of SIRT1 activity in adipose tissue provokes obesity-mediated chronic inflammation and systemic insulin intolerance,

which may lead to lower HIF-2α activity (Gillum et al., 2011; Schug et al., 2010; Yoshizaki et al., 2009; Yoshizaki et al., 2010). Another possibility for incompetence of HIF-2α to induce ARG1 in obese adipose tissue might be resulted from reduction of Th2 cytokines. It has been reported that alternactive activation of macrophages by Th2 cytokines enhances ARG1 expression in HIF-2α-dependent and independent manner (Modolell et al., 1995; Munder et al., 1998; Takeda et al., 2010). Compared to lean animals, in obese, it has been shown that the level of Th2 cytokines is decreased in obese adipose tissue due to decrease of Th2 cytokine-expressing cells such as eosinophils (Wu et al., 2011), which may lead to suppress ARG1 expression. On the other hand, relatvely increased Th1 cytokines such as interferone-γ (IFN-γ) in obese adipose tissue potentiate pro-inflammatory responses (Chawla et al., 2011; Odegaard and Chawla, 2011; Rocha et al., 2008), whose levels might be far behind inability of HIF-2α on ARG1. Moreover, we cannot rule out the possibility that the expression of ARG1 *in vivo* may require for accessory co-factors which appears to be suppressed in obese adipose tissue. Thus, it is feasible that elevated macrohpage HIF-2α activity would be incompetent to overcome augmented pro-inflammatory microenvironments of obese adipose tissue where adipocytes actively cross-talk with ATMs to amplify inflammatory responses.

In conclusion, our data suggest that macrophage HIF-2α would act as a resolving power against adipose tissue inflammation in response to metabolic changes, which leads to keeping homeostatic function of ATMs for healthy adipose tissue remodeling (Fig. 33). In this regard, maintaning of proper HIF-2α activity

Figure 33. Proposed model for role of macrophage HIF-2α in inflammatory response and insulin resistance in obese adipose tissue. In obesity, pro-inflammatory responses between adipocytes and macrophages make positive-feedback. Here, arginase 1 (ARG1) induction by macrophage HIF-2α activation is suggested to suppress vicious cycle of inflammatory responses and to resolve adipose tissue inflammation in response to metabolic stress.


Pro-inflammatory responses
Insulin resistance
FFAs, chemokines
Nitric oxide
TNFα, IL-1β, IL-6
Classically activated
M1 macrophage
Inflamed hypertrophic adipocytes
HIF-2α
ARG1
Alternatively activated
M2 macrophage

would be crucial to prevent adipose tissue dysregulation in obesity, implying that enhacing HIF-2α activity in ATMs might be attractive approaches to treat against obesity-induced metabolic disorders.

CONCLUSION & PERSPECTIVES

Hypoxia-inducible factor 2α (HIF-2α) is one of key transcription factors to mediate hypoxic responses. Emerging evidence has suggested that adipose tissue hypoxia is closely associated with adipose tissue remodeling accompanied chronic inflammation and macrophage accumulation in obesity. To establish the patho-physiological roles of HIF-2α on adipose tissue in obesity, I have investigated the functional role of HIF-2α with regard to angiogenesis and inflammation in adipocytes and macrophages, composing two major cell types of adipose tissue. In summary, I have reached the conclusion that HIF-2α has pro-angiogenic and anti-inflammatory roles in adipose tissue against metabolic disorders such as obesity.

1. HIF-2α and adipose tissue angiogenesis

In obesity, insufficient vascular network and/or blood flow provoke adipose tissue hypoxia, which raises the incidence of adipose tissue inflammation and insulin resistance, causing metabolic complications (Sun et al., 2011; Sung et al., 2013). Thus, for metabolically healthy adipose tissue expansion, sufficient development of adipose tissue vasculature via angiogenesis appears to be needed in the progress of obesity. In this study, I have demonstrated that HIF-2α is one of the key transcription factors for angiogenesis in rapidly expanding adipose tissue in obesity. Several lines of observations have proposed that increased HIF-2α in adipose tissue might exert pro-angiogenic effects. First, the expression of pro-

angiogenic genes including VEGFα and ADM was stimulated by HIF-2α overexpression in adipocytes and macrophages, which may lead to modulation of the angiogenic and survival activities in endothelial cells. Second, HIF-2α-overexpressing adipocytes increased macrophage recruitment, which might eventually support adipose tissue angiogenesis. According to *in vitro* co-culture data, macrophages recruited as neighborhood of adipocytes showed transcriptional up-regulation of IGF-1 and PDGFβ, which are key components of pro-angiogenic factors. In addition, macrophages exhibit a pro-angiogenic phenotype by sensitively and highly inducing the expression of VEGFα and ADM in response to chronic hypoxia. These results propose that macrophages recruited into adipose tissue efficiently would support angiogenesis at local microhypoxic areas. Third, the expression of MMPs genes such as MMP-3, MMP-9, and MMP-13 was significantly induced in HIF-2α-overexpressing adipocytes. For the initiation of angiogenesis, the breakdown and degradation of ECM are crucial steps, which are well-known to be mediated by MMPs in solid tumor or wound healing (Pepper, 2001; Yancopoulos et al., 2000). In angiogenesis, extracellular proteolysis by MMPs has been also implicated in the recruitment of specific cells, including progenitor cells for blood vessel formation and macrophages for the supplementing of additional pro-angiogenic factors (Pepper, 2001; Yancopoulos et al., 2000). Moreover, MMP-9 is able to release matrix-bound VEGFα, implying that increased MMP-9 by HIF-2α would directly intensify pro-angiogenic signaling (Bergers et al., 2000). Last, NO induced by adipocyte HIF-2α may contribute to VEGFα production

and vasodilatation as in many types of tumor cells (Singh and Agarwal, 2007). On the other hand, when HIF-2α is overexpressed in macrophages, NO production is decreased by ARG1 activation, which did not disrupt transcriptional regulation of pro-angiogenic factors. Macrophage ARG1 is reported to contribute to collagen synthesis via promotion of prolin production (Fujisaka et al., 2009; Lumeng et al., 2007a), which may lead to the repair and stabilization of remodeled adipose tissue through collagen supply and deposition after angiogenesis rather than direct participation in angiogenesis. Consequentially, activated HIF-2α appears to orchestrate the cross-talk between adipocytes and macrophages, thus synergistically promoting adipose tissue angiogenesis.

2. HIF-2α and adipose tissue inflammation

In obesity, increased ATMs have been considered to play an important role in the mediation of adipose tissue inflammation, in which the population of ATMs inclines toward a pro-inflammatory phenotype because of the robust elevation of M1 ATMs recruitment (Donath and Shoelson, 2011; Olefsky and Glass, 2010; Wellen and Hotamisligil, 2003). However, paradoxically, the resolution of pro-inflammatory response appears to be an another functional role of macrophages via alternated activation (Fujisaka et al., 2009; Lumeng et al., 2007a). In this work, I have demonstrated that macrophage HIF-2α participates in the amelioration of adipose tissue inflammation and insulin resistance in obesity. In macrophages, overexpression of HIF-2α stimulated ARG1 expression, which reduced the levels of

NO and mRNA expression of pro-inflammatory cytokine genes such as TNFα, IL-6, and IL-1β. In addition, macrophage HIF-2α protected adipocytes against inflammatory signals and insulin resistance driven by cross-talk between adipocytes and macrophages. As a result, these data suggest that macrophage HIF-2α activation would be one of the resolving pathways in obese adipose tissue. In macrophages, several underlying mechanisms of the anti-inflammatory effects of HIF-2α via ARG1 induction have been proposed. One of these possible mechanisms may be related to the scavenging of reactive nitrogen species, which could mediate pro-inflammatory signaling or readily react with reactive oxygen species to boost pro-inflammatory responses (Fujisaka et al., 2009; Lumeng et al., 2007a). The second possible explanation for the anti-inflammatory ability of macrophage HIF-2α may be related to ARG1-mediated polyamine production, which is reported to down-regulate the release of pro-inflammatory cytokines (Bronte and Zanovello, 2005). Furthermore, *in vivo*, ARG1 activation has been shown to lead to T cell suppression via the consumption of L-arginine from the extracellular environment (Bronte and Zanovello, 2005), which may suppress adipose tissue inflammation involving in various immune cells including T cells in obesity.

On the other hand, the role of adipocyte HIF-2α on adipose tissue inflammation is controversial. Recent data indicate that adipocyte HIF-2α seems to participate partly in inflammatory response such as macrophage recruitment and the induction of pro-inflammatory genes including iNOS, IL-6, and SAA, which may lead to adipocyte dysfunction through interaction with augmented pro-inflammatory

environments of obese adipose tissue, although no such detrimental effects has been observed *in vitro* culture condition. However, adipocyte HIF-2α also seems to induce anti-inflammatory cytokine, IL-10, counteracting pro-inflammatory cytokines. In addition, in macrophages recruited by adipocyte HIF-2α, the pro-inflammatory activities of macrophages could be suppressed by macrophage HIF-2α. Furthermore, HIF-2α is also involved in the regulation of pro-angiogenic factors including VEGFα, ADM, ANGPTL4, and PAI-1, by which angiogenic activation may inversely suppress inflammatory response via preventing adipose tissue hypoxia *in vivo* (Sun et al., 2011; Sung et al., 2013). To clearly demonstrate the *in vivo* role of adipocyte HIF-2α, it is demanded to develop a mouse model in which HIF-2α is overexpressed or knocked-out in adipocytes. Nevertheless, in *in vivo* data using HIF-2α heterozygote mice, HIF-2α$^{+/-}$ mice exhibited phenotype that is susceptible to adipose tissue inflammation and insulin resistance in DIO, implying that, at least, HIF-2α in whole body is expected to contribute to resolve adipose tissue inflammation in response to metabolic challenges in DIO.

3. Regulation of HIF-2α in adipose tissue

The HIFα family is primarily regulated by oxygen-dependent post-translational stabilization. In addition, recent data have indicated that control of HIF-2α expression can be selectively regulated at the level of transcription, but relatively little is known about the transcriptional regulation of HIF-2α (Keith et al., 2012). In this work, I have demonstrated that the expression level of HIF-2α mRNA

is significantly induced in adipocytes of mice fed HFD only for 1 week. According to *in vitro* data, the transcriptional up-regulation of HIF-2α is associated with adipogenic and hypertrophic stimulation. However, the pathways involved in induction of HIF-2α mRNA have not been clearly defined. In previous studies, transcription factors such as C/EBPβ/δ and Sp1/Sp3 are reported to be related to the induction of HIF-2α mRNA during adipogenesis, but the precise mechanisms are unclear (Shimba et al., 2004; Wada et al., 2006). Although further studies are required for clarification of which pathways could regulate the expression of HIF-2α in adipocytes, the activation of protein kinase C (PKC), serine/threonine kinase related to various signal transduction, and sterol regulatory element-binding protein 1 (SREBP1), key transcription factor related to adipogenesis and lipogenesis, is suspected to be one of the possible pathways involved in transcriptional regulation of HIF-2α. During this study, I observed that the mRNA level of HIF-2α seemed to be increased by PKC activator, PMA, while is decreased by broad spectrum PKC inhibitor. Also, conserved SREBP1 binding elements (SRE) could be found in the 0.5 kb region of mouse, rat, and human HIF-2α promoter.

Interestingly, I noted that the expression of two enzymes, iNOS and ARG1 that competitively metabolize L-arginine, was regulated by HIF-2α in adipocytes and macrophages, respectively. Adipocyte HIF-2α induced the expression of iNOS gene, whereas macrophage HIF-2α stimulated the expression of ARG1. Although the molecular mechanism for cell type-specific regulation of HIF-2α target genes is not well-defined, previous study has suggested that the status of chromatin

structures at HIF response elements (HREs) in HIF-2α target gene promoters might be important for cell type-specific regulation of its target genes (Schodel et al., 2011). For instance, when HREs of its target gene promoters is at the state of open chromatin structures, HIF-2α mainly binds to there. In addition, HIF-2α is unable to change directly the chromatin structures around HREs. Therefore, it is likely that HREs of ARG1 promoter in adipocytes might be hidden at the state of closed chromatin structure, interfered with the binding of HIF-2α, whereas, the binding of HIF-2α to iNOS promoter in macrophages might be inhibited by closed chromatin structures around its HREs. However, to more clarify the molecular mechanisms for cell-type specific regulation of HIF-2α target genes, further studies are required.

4. Regulation of HIF-2α pathway in obese adipose tissue

In obese adipose tissue, the increased HIF-2α has been appeared to regulate the anti-inflammatory and pro-angiogenic activity. Overexpression HIF-2α in adipocytes and macrophages stimulated the mRNA expression of pro-angiogenic genes. In addition, HFD-fed HIF-2α$^{+/-}$ mice exhibited augmented pro-inflammatory responses in adipose tissue. However, it is feasible that elevated macrohpage HIF-2α in severe obesity would be incompetent to overcome augmented pro-inflammatory and hypoxic microenvironments of obese adipose tissue. Recent studies have proposed that SIRT1 deacetylates conserved lysine residues of HIF-2α, and this deactylation enhances HIF-2α transcriptional activity (Cho and Lumeng, 2011; Dioum et al., 2009). However, it is well-established that decrease of SIRT1

activity in adipose tissue stimulates obesity-mediated chronic inflammation (Gillum et al., 2011; Schug et al., 2010; Yoshizaki et al., 2009; Yoshizaki et al., 2010). In addition, SIRT1 knock-out mice shows the reduction of blood vessel density and several angiogenic factors (Xu et al., 2012). Therefore, it is likely that deficiency of SIRT1 activity in obesity may remain the excessive acetylation of HIF-2α in adipose tissue, which may lead to lower HIF-2α activity in spite of elevated HIF-2α expression. However, additional investigations for the regulation of HIF-2α activity in obese adipose tissue are warranted. Moreover, we cannot rule out the possibility that the expression of target genes by HIF-2α *in vivo* may require for accessory co-factors which might be suppressed in obese adipose tissue.

In conclusion, I suggest that in adipose tissue, the pro-angiogenic and anti-inflammatory functions of HIF-2α may be involved in adipose tissue remodeling in obese state. This study indicates that increased adipocyte HIF-2α in early stage of HFD may provide pro-angiogenic factors for adipose tissue angiogenesis. Furthermore, macrophage HIF-2α would resolve local inflammation in adipose tissue in reponse to metabolic changes. Taken together, the activation of HIF-2α in adipocytes and ATMs would be crucial for maintaining the homeostasis of adipose tissue inflammation and vasculature against metabolic stresses, which would contribute to prevent severe insulin resistance in obesity. Therefore, it is feasible to speculate that the enhacing HIF-2α activity in adipose tissue might be alternative approaches to treat against obesity-induced metabolic disorders.

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국문 초록

우리 몸의 지방조직은 영양상태에 따라 그 크기가 빠르게 증가하거나 감소하는 특성을 갖는다. 이 과정에서 양분 과다 섭취에 의한 지방조직의 급격한 팽창은 기존 혈관 구조와의 불균형으로 인해 국지적으로 저산소 상태를 유발한다. 현재 비만에서 관찰되는 지방조직 내 저산소증(hypoxia)은 염증반응 (inflammatory response)의 원인으로 주목 받고 있다. 이 과정에서 hypoxia-inducible factor (HIF) family는 지방조직에서 저산소 상태를 인지하고 관련 유전자의 발현을 조절할 수 있는 중요한 전사인자로 제시되고 있다. 예를 들어 비만 지방조직에서 HIF-1α의 활성화는 지방조직의 혈관신생 (angiogenesis) 촉진뿐만 아니라 만성적 염증반응 및 섬유화, 인슐린 저항성과 밀접하게 연관되어 있다고 보고되어 있다. 그럼에도 불구하고 HIF family 중 하나인 HIF-2α의 병리생리학적 기능에 대한 연구는 잘 이루어 지지 않은 실정이다. HIF-2α에 대한 지금까지 연구들은 HIF-1α와 HIF-2α의 구조적 기능적 유사성에도 불구하고 HIF-2α가 HIF-1α와는 구분되는 혹은 상반되는 생리적 기능을 갖고 있음을 보여주었다. 따라서 지방조직에서의 HIF-2α 역시 비만 진행과정에서 자신만의 독특한 역할을 가질 것으로 제안된다.

본 연구를 통해, 지방세포의 HIF-2α 활성화가 지방조직에서 혈관생성인자 (pro-angiogenic factor)의 공급에 중요할 수 있음을 밝혔다. 흥미롭게도 1주의 단기간 고지방 식이 섭취만으로도 생쥐의 지방세포에서 HIF-

2α의 발현이 증가하였다. 이렇게 증가한 지방조직 HIF-2α의 역할을 규명하기 위해, 지방세포에서 HIF-2α를 과발현 시킨 결과 VEGFα, AMD, ANGPLT4, PAI-1과 같은 혈관생성인자의 전사 활성이 증가하는 것을 관찰하였다. 이와 함께, 지방세포에서의 HIF-2α에 의한 iNOS의 증가는 대식세포 모집 (macrophage recruitment)을 촉진시켰다. 실험결과 대식세포는 지방세포와의 상호작용을 통해 IGF-1, PDGFβ와 같은 혈관생성인자를 발현할 뿐만 아니라 지방세포 보다 빠르게 저산소 상태를 인지하고 VEGFα 및 AMD의 발현을 증가시켰다. 따라서 이렇게 모집된 대식세포는 비만 개체의 지방조직에서 혈관생성인자 공급의 또 다른 한 축을 이룰 것으로 생각된다.

또한 지방 조직 거식세포 (adipose tissue macrophages; ATMs)에서 증가한 HIF-2α가 비만에서 보이는 지방조직 염증반응 억제하고 인슐린 저항성 개선함을 밝혔다. 대식세포에 HIF-2α를 과발현 시키면 산화 질소 (nitric oxide)의 생성과 염증성 사이토카인 (inflammatory cytokine)의 발현이 감소되었는데 이는 대식세포 HIF-2α에 의해 arginase 1의 발현이 촉진되었기 때문으로 생각된다. 실제로 HIF-2α가 과발현 된 대식세포를 3T3-L1 지방세포주와 공동배양 시킨 결과, 대식세포에 의해 유도되는 지방세포에서의 염증 반응 및 인슐린 저항성이 억제되는 것을 관찰 할 수 있었다. 반대로 HIF-2α의 발현을 억제한 대식세포를 지방세포와 공도배양 시키자, 정상 대식세포와 공동배양 할 때 보다 지방산 유래 지방세포 염증 반응이 악화되었다. 더욱이, 게놈상에 HIF-2α 유전자가 한 벌 부족한 HIF-2α

heterozygote 생쥐에 고지방 식이를 섭취시키자 야생형 (wild type) 생쥐에 비해 전염증성 (pro-inflammatory) 대식세포 수의 증가와 함께 지방세포 염증반응 및 인슐린 저항성이 악화되는 것을 관찰하였다. 고지방 식이 상태에서 관찰되는 HIF-2a 부족으로 인한 인슐린 저항성은 clodronate을 이용하여 지방조직에서 대식세포를 제거하자 개선되었다. 따라서, HIF-1α와 달리, 지방조직 대식세포에서 HIF-2α는 비만 개체에서 대사 스트레스로 인해 유발되는 지방 염증반응 및 인슐린 저항성을 해결하는데 중요한 역할을 할 것으로 새롭게 제안된다.

본 연구의 결과를 종합적으로 살펴볼 때, 최소한의 염증반응이 관찰되는 초기 비만에 있어, HIF-2α는 신생혈관생성을 촉진하고 과도한 염증반응을 제어함으로써 대사환경 변화에 맞춰 지방조직 리모델링을 조절할 것으로 생각된다. 따라서 HIF-2α의 활성 제어를 통한 지방조직의 염증반응 및 혈관 생성의 항상성 유지는 대사 스트레스에 대항하여 지방조직을 건강하게 유지하는 생리 기전으로 제안된다.

주요어: Hypoxia-Inducible Factor 2α, 지방조직 저산소증, 지방조직 염증반응, 지방조직 리모델링, 지방조직 대식세포, 비만, 인슐린 저항성

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